PATHOPHYSIOLOGY OF CEREBELLAR-INDUCED MOTOR DISORDERS

by

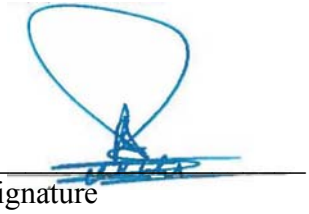
Esra Tara

Candidate:

Signature

Esra Tara
Name

Thesis Advisor:



Signature

Kamran Khodakhah, Ph.D.
Name

Professor, Dominick P. Purpura
Department of Neuroscience
Title

Submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy
in the Graduate Division of Medical Sciences

Albert Einstein College of Medicine
Yeshiva University
New York
August 2012

ABSTRACT

Pathophysiology of cerebellar-induced motor disorders

Esra Tara

The cerebellum is the brain region that ensures the proper timing and coordination of movements. Not surprisingly, the best appreciated symptom of cerebellar dysfunction is ataxia, a loss of motor coordination. Mounting evidence however, implicates the cerebellum in a host of motor disorders characterized by involuntary muscle contractions such as dyskinesia and dystonia. Given the commonly ascribed function of the cerebellum, that it coordinates movements but does not initiate them, the fact that it not only plays a role in but often is the source of dysfunction in many such disorders is counterintuitive.

Episodic ataxia type-2 (EA2) is one such condition that results from mutations in the gene encoding P/Q-type voltage-gated calcium channel. In humans, the phenotype is characterized by a moderate basal ataxia as well as paroxysmal attacks of severe ataxia and dyskinesia triggered by caffeine, ethanol and psychological stress. The *tottering* mouse is a faithful model of EA2 and mimics the human EA2 condition both genetically and phenotypically.

In the first study, we used the spectrum of motor dysfunction displayed by the *tottering* to delineate the cerebellar output patterns associated with the disorder's episodic phenotype. Extracellular recordings from single Purkinje cells in the awake *tottering* in

the presence and absence of dyskinesia episodes revealed that during attacks, their simple spike activity is significantly altered and characterized by erratic firing and bursts of high frequency. This activity pattern was necessary and sufficient for the expression of attacks, and resulted in an aberrant cerebellar output. Moreover, we found the severity of the *tottering*'s motor symptoms to be correlated with the extent of Purkinje cell burst firing and the noise in the consequent cerebellar output.

Episodic channelopathies such as EA2 are characterized by a normal or mildly symptomatic baseline phenotype, interrupted by attacks of severe symptoms. A common feature of these disorders is that in all of them, attacks are induced by a same set of triggers, caffeine, alcohol, and stress. This suggests the existence of a shared mechanism for attack initiation. In the next study, we used the *tottering* as a model for episodic neurological disorders and combined electrophysiological techniques with a pharmacological approach to examine the pathway by which stress triggers attacks of dyskinesia.

We found blocking cerebellar noradrenergic receptors to prevent stress from triggering an attack and the release of norepinephrine from the locus ceruleus to be necessary and sufficient to induce the transient motor symptoms. Further examination revealed the norepinephrine-induced inhibition of small-conductance calcium-activated potassium channels as a potential mechanism underlying the dysfunctional firing patterns observed *in vivo*.

To further examine the cerebellar output patterns associated with dysfunctional motor phenotypes, we examined them in a mouse model of spinocerebellar ataxia as well as the *car8* mutant mouse, whose phenotype is characterized by a severe form of ataxia and appendicular dystonia. In both mice, we found the extent of aberrant output from the cerebellum to predict the severity of the motor dysfunction, which could be alleviated by forcing Purkinje cells to fire more regularly. We then recorded the activity of DCN neurons in wild type mice while they performed a variety of cerebellar-dependent motor tasks. The erratic firing pattern observed in mutants never occurred in wild types during any of the physiologically relevant conditions, further substantiating a causal relationship between aberrant cerebellar output and motor dysfunction.

Taken together, the results from this thesis support the conclusion that cerebellar ataxia is caused by diminished information content of the cerebellar output signals, whereas dystonia and dyskinesia result from bursting activity in the output nuclei acting as erroneous command signals to cause aberrant muscle contractions. In EA2, stress transiently triggers such a change in cerebellar output via norepinephrine-induced burst firing at the level of Purkinje cells.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Kamran Khodakhah, who is not only an amazing mentor but a dear friend as well. I could not have asked for a more inspirational, supportive and patient role model. Thank you for your guidance and mentorship, and for always asking me for more than I thought I could achieve. It has truly been an honor and privilege to learn from you, I would not be the same person today if it wasn't for you.

I am grateful for the chance to have been a part of Khodakhah Lab. Many thanks to all my lab mates, a group with a distinct personality: amicable, fun and always available to teach and provide thorough feedback. Thank you for putting up with me, for all your help and last but not least, for patiently scoring my videos! Thank you Sung-Min for being so forthcoming with your project and Christopher for your enthusiastic help with mine. It has been a joy to learn from and work alongside you. Thank you Johanna for always being there to commiserate and for patiently answering all my questions throughout the writing of this thesis.

I would also like to thank my examiners, Drs. Jose Luis Peña, Henry Paulson, Adam Kohn, Saleem Nicola and Don Faber for reviewing my thesis and providing thoughtful and detailed comments. Many thanks to Drs. Ellen Hess for helpful discussions and feedback and Roy Sillitoe for allowing me to be a part of a great project.

Thank you Rana, not only for your help throughout this thesis but for being the best friend I could ever hope for. A great many thanks to my mom, for always being there to listen and support me through the best and the worst. Thank you dad for never failing to guide me and always having the right answer. You are my inspiration.

I cannot thank my husband, without whom this thesis would not have been possible, enough. I am grateful to have someone who believes in me much more than I ever did myself. Thank you for your eternal patience and optimism, thank you for always listening and supporting me. Thank you for caring so much. I love you.

To Ziad,
with my gratitude.

LIST OF FIGURES

LIST OF ABBREVIATIONS

aCSF – artificial cerebro-spinal fluid
AHP – afterhyperpolarization
CA8 – human carbonic anhydrase-related protein VIII
car8 – mouse carbonic anhydrase-related protein VIII
$CdCl_2$ – cadmium chloride
CHZ – chlorzoxazone
CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione
CV – coefficient of variation
DCN – deep cerebellar nuclei
DYTCA – dystonia with cerebellar atrophy
EA – episodic ataxia
EPSCs – excitatory post-synaptic-like currents
EPSPs – excitatory post-synaptic potentials
FR – firing rate
GABA – γ-Aminobutyric acid
ISI – interspike interval
IPSCs – inhibitory postsynaptic currents
Klhl1 – Kelch-like 1
$Klhl1^{flox/flox}$ – wild-type littermates
mIPSCs – miniature IPSCs
PSTH – post-stimulus time histograms
R – Pearson's correlation coefficient
RNA – ribonucleic acid
SCA – spinocerebellar ataxia
sIPSCs – spontaneous inhibitory post-synaptic currents
SK – small conductance calcium-activated potassium
sp/s – spikes per second
UBCs – unipolar brush cells

TABLE OF CONTENTS

CHAPTER I:

INTRODUCTION

OVERVIEW

The symptoms of cerebellar dysfunction have been well characterized and the cerebellar neurological examination as it is used today has been established for more than a century. The genetic mutations underlying most inherited cerebellar syndromes have also been identified, but an understanding of the mechanisms by which dysfunction in the cerebellum translates into the expression of motor symptoms is lacking. The overall goal of this thesis is to contribute to the understanding of the pathophysiology underlying motor syndromes associated with the cerebellum. To this end, cerebellar activity in mice genetically modeling specific human inherited motor disorders was examined in order to gain insight into 1) the disease mechanism unique to each disorder by virtue of the specific mutation causing it and 2) the output patterns by which the cerebellum generates motor dysfunction.

Much of our current understanding of cerebellar function emerged from the examination of its dysfunction, either experimentally induced in animals or as a result of diseases and accidents injuring it in humans. The following introduction provides a summary of the knowledge that was so gained with regard to cerebellar function in health and its contribution to disease. The first part focuses on seminal experiments in which the outcome of losses in cerebellar function was observed to infer what its function is currently thought to be. It is also these same observations that revealed the nature of symptoms, the presence of which today define a disorder as being cerebellar in origin. The second part summarizes anatomical and physiological information pertinent to the studies in the subsequent chapters. This is followed by a description of inherited disorders of the cerebellum with an emphasis on the physiological underpinnings of their

associated symptoms. In the last part, the clinical and experimental evidence implicating cerebellar dysfunction in the production of hyperkinetic motor symptoms is delineated.

A. CEREBELLAR FUNCTION AND PROPERTIES

The first approach to the study of the function of an organ has historically been to observe the outcome when that organ is removed first as a whole, and then in its parts. Given its easily accessible localization, this has especially been true for the cerebellum. Our current understanding of cerebellar function is by and large shaped by studies of surgical and accidental lesions in animals and humans. The characterization of the primary function of the cerebellum as being motor in nature is credited to experiments performed by Luigi Rolando in the early nineteenth century. Based on his observation that among all the regions of the brain, galvanic stimulation of the cerebellum caused the most violent muscle contractions, and its ablation led to difficulties in performing movements, Rolando concluded that the cerebellum is the site of movement initiation (Rolando, 1809).

Although he correctly identified a role for the cerebellum in motor behavior, Rolando's ultimate conclusion, that it is the center for voluntary movement, was inaccurate. The foundations of the now widely held view that the cerebellum is a coordinator rather than an initiator of movements dates back to a seminal series of experiments conducted by Flourens in 1824. He noted that upon surgically removing the cerebellum in a wide range of animals, the ability to generate movements remained but the animals moved about clumsily, as if drunk. This led him to the famous conclusion "in the cerebellum resides a function the nature of which had so far not been revealed by physiology, and that consists of the coordination of movements" (Flourens, 1824).

Similar experiments subsequently confirmed that even the complete removal of the cerebellum does not lead to paralysis and led to the consensus that the function of the cerebellum is not to instigate but to coordinate movement. Flourens additionally noted that when he removed only a small portion of the cerebellum, the resulting deficits progressively recovered (Flourens, 1824). When he removed the entire cerebellum however, certain symptoms either never fully recovered or recovered only much later. This was confirmed later on in a classic experiment done by Luciani, who noted that if he removed a small fraction of a dog's cerebellum, the resulting motor symptoms would fully recovered within a matter of weeks. However, when upon recovery he placed a second lesion adjacent to the original one, the resulting deficits would be twice as severe as the first one, indicating that compensation for the first lesion occurred within the cerebellum itself (Luciani, 1891). From this experiment he concluded that the cerebellum is a highly plastic structure that is capable to learn.

At the present day the function of the cerebellum is still a topic of active debate. However, owing to experiments similar to the ones above, there is a general consensus that with regard to motor function the fundamental functions of the cerebellum are to 1) participate in the maintenance of balance and posture 2) integrate sensory and cortical input to mediate the precise execution of voluntary movements, 3) enable the skilled performance of movements by learning motor patterns (Ito, 1984).

1. PATHWAYS FOR MOTOR CONTROL

Movements both voluntary and involuntary result from the contraction of muscle. They are controlled by a spinal as well as a supraspinal motor control system. The cerebellum is one of multiple components that together form the supraspinal motor

control system and its connections to other elements of this system provide the framework for its function and its dysfunction. The supraspinal control of movement occurs via descending pathways, which can broadly be divided into two: 1) brainstem pathway (lateral and ventromedial) and 2) corticospinal pathway (Afifi and Bergman, 1997; Figure 1).

Each descending pathway controls movements by making monosynaptic, disynaptic or polysynaptic connections to motor neurons (Afifi and Bergman, 1997). The brainstem pathways originate mainly in the pontine nuclei, red nucleus, reticular formation, vestibular nuclei and superior colliculi and project to interneurons controlling limb and axial muscles (Afifi and Bergman, 1997). The corticospinal pathway originates mainly in the pre-motor, supplementary and motor cortices and projects to motor neurons or to interneurons innervating them. Corticospinal tract collaterals additionally project to the brainstem and influence the descending pathways originating at this location as well (Afifi and Bergman, 1997). The corticospinal and brainstem thus form the two major motor control loops. Additionally, the basal ganglia, thalamus and cerebellum influence motor control by each forming sub-loops within these two major loops (Afifi and Bergman, 1997; Figure 1)

The cerebellum receives input from and projects to both motor control loops. Afferents originating in brainstem nuclei relay information from both descending (from cortical regions to muscles) and ascending (from the spinal cord to cortical regions) tracts (Ito, 1984). Cerebellar efferents project to the brainstem tracts via monosynaptic connections to brainstem nuclei, and to the corticospinal tract via disynaptic pathways through the thalamus and basal ganglia (Cohen et al., 1958; Asanuma et al., 1983b, a;

Hoshi et al., 2005). In addition, the cerebellum forms a monosynaptic connection with the spinal cord (Matsushita and Hosoya, 1978; Asanuma et al., 1980; Bentivoglio, 1982). By receiving motor and sensory information from the cerebral cortex and vestibular, acoustic, visual and somesthetic information relating to the execution of a movement, the cerebellum is informed of both the intent to execute a movement, the parameters relating to its execution and the context within which it is executed. In turn, the cerebellum provides information to cortical areas that initiate movements, and to muscles effectuating them (Ito, 1984).

2. INPUT AND OUTPUT

Two major afferents supply input to the cerebellum. An immense variety of cortical and sensory inputs arrive via mossy fibers, the cell bodies of which are located mostly in the spinal cord and various brainstem nuclei. In stark contrast, climbing fibers provide input originating in only one location, the inferior olive (Ito, 1984).

The cerebellum is anatomically subdivided into two regions, the cortex and the nuclei. Input to the cerebellum ultimately converges onto the cerebellar cortex, and output from the cerebellum departs from the deep cerebellar nuclei (DCN). Within the cerebellar cortex, Purkinje cell bodies are arranged in a single sheet, forming a layer that separates the innermost granule cell layer from the outermost molecular layer (Palkovits et al., 1971; Palay and Chan-Palay, 1974). Cerebellar cortical cells are housed in either of these two layers, granule cells and interneurons (Golgi, unipolar brush, Lugaro) in the deepest layer and molecular layer interneurons (stellate and basket) in the most superficial one (Palay and Chan-Palay, 1974) (Figure 2).

All inputs to the cerebellum ultimately converge onto Purkinje cells, with information generally flowing in a feedforward manner. Thus, mossy fibers excite granule cells, which excite Purkinje cells, the sole output from the cortex. In addition, feedforward inhibition of granule cells is mediated by the mossy fiber input to Golgi cells and that of Purkinje cells by the excitatory action of granule cells on molecular layer interneurons. Purkinje cell activity is additionally modulated by the action of noradrenergic afferents from the dorsal and ventral locus ceruleus, the majority of which form contacts with Purkinje cell dendrites in the molecular layer and granule cell glomeruli and a minority targeting DCN neurons (Bloom et al., 1971). Purkinje cells form inhibitory connections with DCN neurons, which provide the ultimate output from the cerebellum (Ito et al., 1970) . In addition to the DCN, Purkinje cells within a small portion of the cerebellar cortex (flocculonodular lobe) directly project out of the cerebellum to the lateral vestibular nucleus in the brainstem (Haines and Dietrichs, 2012).

The output from the cerebellum is via four nuclei embedded in a compact mass of myelinated axons (white matter core) underneath the cerebellar cortex. These are the interposed, fastigial (itself composed of the globose and emboliform nuclei) and dentate (lateral in rodents) nuclei. The axons of large excitatory DCN neurons project out of the cerebellum and send collaterals to brainstem nuclei, red nucleus, thalamus and basal ganglia (Asanuma et al., 1974; Chan-Palay, 1977b; Hoshi et al., 2005; Haines and Dietrichs, 2012). The small inhibitory neurons are thought to comprise small local circuit neurons and projection neurons exclusively targeting the inferior olive (Matsushita and Iwahori, 1971b; Chan-Palay, 1977a). A fraction of DCN neurons make monosynaptic connections with the spinal cord (Matsushita and Hosoya, 1978) and all send axon

collaterals to the area of the cerebellar cortex from which they receive input (Gould and Graybiel, 1976).

The DCN receive converging inhibitory input from Purkinje cells as well as excitatory ones from mossy and climbing fiber collaterals (Matsushita and Iwahori, 1971a; Chan-Palay, 1977a). In this way, the mossy fiber-parallel fiber system regulates the excitability of the cerebellar nuclei, either directly via mossy fiber collaterals or through their input to the cerebellar cortex.

3. PHYSIOLOGY

3.1 Purkinje cells

Purkinje cells are strategically placed to act as the main computational element of the cerebellar cortex, integrating an enormous amount of cortical and sensory input and relaying it to output neurons. They are spontaneously active and in the absence of synaptic inputs tonically fire action potentials called simple spikes with an average rate of 50 spikes per second (Raman and Bean, 1999a). The coefficient of variation of interspike intervals of their intrinsic pacemaking is approximately 0.05, indicating a highly regular pattern of activity (Hausser and Clark, 1997; Walter et al., 2006).

During the execution of various movements such as self-initiated limb movements, locomotion and smooth-pursuit eye movements, the firing rate increases and decreases from this average rate in response to synaptic input and in correlation with the contraction of muscles necessary for the execution of the movement (Thach, 1968, 1970; Lisberger and Fuchs, 1978; Ito, 1984). This modulation correlates with several movement parameters, including (depending on the movement) its velocity and direction (Thach, 1968; Lisberger and Fuchs, 1978). Thus, by encoding ascending sensory inputs related to

the execution of movements and descending cortical inputs related to motor command signals, Purkinje cells provide the framework for the signals required to execute coordinated movements (Ito, 1984). They are thought to do so by generating precise timing signals for the activation and inhibition of appropriate agonist and antagonist muscles implicated in the execution of a given movement as changes in the rate and the pattern of their firing (Ito, 1984). Accordingly, in humans, the degeneration of Purkinje cells associated with various mutations has devastating effects on motor coordination (Paulson, 2009; Seidel et al., 2012) and similarly mutant mice lacking Purkinje cells are severely ataxic (Mullen et al., 1976).

Given the information contained in their firing pattern, alterations in the excitability of Purkinje cells would be predicted to significantly impair motor function. Indeed, pharmacologically altering the properties of various cerebellar ion channels that contribute to the excitability of Purkinje cells results in motor dysfunction in animals (Sausbier et al., 2004; Calderon et al., 2011; Fan et al., 2012). Moreover, mutations in various inherited forms of cerebellar dysfunction have deleterious consequences on the physiology of Purkinje cells (Pietrobon, 2002; Walter et al., 2006; Shakkottai et al., 2011). In order to understand cerebellum's dysfunction, it is important to understand the properties of the components that make up its circuitry. This is especially relevant with regard to Purkinje cells given that their malfunction is a common theme among various mutations.

The tonic activity of Purkinje cells is orchestrated by the interplay of several conductances. It is spontaneously maintained in part due to the presence of fast-deactivating potassium conductances that do not allow Purkinje cells to hyperpolarize

steeply as well as a resurgent sodium current which, between two spikes, depolarizes the membrane potential towards threshold (Raman and Bean, 1999b). An action potential waveform additionally evokes calcium currents, which are also required for the maintenance of Purkinje cell tonic activity (Raman and Bean, 1999b).

3.1.1. P/Q-type voltage-gated calcium channels

The main voltage-gated calcium channels activated by an action potential waveform in Purkinje cells are T- and P/Q-type (Raman and Bean, 1999b). The effect of calcium influx during the repolarization phase, the bulk of which is mediated by P/Q-type channels, is not an inward current as would be expected from the influx of calcium, but an outward one (Raman and Bean, 1999b). This is due to the highly efficacious coupling of calcium entry through P/Q- channels to the activation of calcium-activated potassium (K_{Ca}) channels (Womack et al., 2004). As a result of this coupling, calcium influx is in effect outweighed by the potassium conductance it triggers. This outward current contributes to the action potential afterhyperpolarization (AHP) (Edgerton and Reinhart, 2003; Womack et al., 2009). Thus, block of calcium entry prevents tonic firing, not because of the absence of a depolarizing calcium current, but due to the lack of the hyperpolarizing outward current mediated by K_{Ca} channels driving the cell into depolarization block.

3.1.2. Calcium-activated potassium channels

Two types of potassium conductance activate via calcium entry through P/Q-channels, large conductance voltage-dependent BK channels and small-conductance voltage-independent SK channels (Womack and Khodakhah, 2002; Edgerton and Reinhart, 2003). BK channels activate rapidly during the repolarization phase and due to

their voltage-dependence, rapidly deactivate as the cell hyperpolarizes. In contrast, SK channels activate much more slowly and due to their voltage-independence, their deactivation is also slow (Edgerton and Reinhart, 2003). Although in Purkinje cells neither channel contributes to action potential repolarization or to the resting membrane potential, their activation is time-locked to action potential firing and the bulk of the current they carry shapes the Purkinje cell AHP (Womack et al., 2009).

In several pacemaking cells, the effect of blocking SK channels produces irregular firing (Hallworth et al., 2003; Deister et al., 2009). In Purkinje cells block of SK channels results in an increase in the firing rate and a decrease in its regularity (Womack and Khodakhah, 2003). This is due to the depolarizing effect of calcium entry through P/Q-channels, which in the absence of the SK conductance, is enough to produce a net depolarization, allowing the cell to reach threshold more rapidly (Womack and Khodakhah, 2003).

The open probability of K_{Ca} channels increases with elevations in cytosolic calcium (Vergara et al., 1998). SK channels assemble as tetramers; they are voltage-independent and gated by submicromolar concentrations of intracellular calcium, local elevations of which increase their open probability (Vergara et al., 1998). Although they are gated by $[Ca^{2+}]_i$, they do not bind to calcium. Rather, their calcium gating is endowed by calmodulin (CaM), which binds to each subunit's CaM-binding domain (Allen et al., 2007). Binding and unbinding of calcium to CaM induces a conformational change, which opens or closes the channel. SK channels constitutively associate with casein kinase 2 (CK2) and protein phosphatase 2A (PP2A), the activation of which respectively increase and decrease the channel's affinity for calcium (Allen et al., 2007). CK2 does

not phosphorylate SK channels; rather it phosphorylates a site on CaM, thereby reducing the channel's affinity for calcium by 5-fold. Subsequent dephosphorylation by PP2A decreases the calcium sensitivity (Allen et al., 2007).

Although SK channels cannot pharmacologically be "activated", there are several agents that shift their affinity for calcium to the low nanomolar range (SK channel "activators") (Pedarzani et al., 2005). They are thought to do so by stabilizing the Ca^{2+}-CaM-SK interaction, and therefore slowing the channel's deactivation (Pedarzani et al., 2005). In Purkinje cells, the effect of increasing SK channels' affinity for calcium using high (nonphysiological) concentrations of activators is a decreased firing rate (Womack and Khodakhah, 2003). This is due to the increase of the outward current mediated by SK channels, which lengthens the AHP and increases the time it takes to depolarize back to threshold (Womack and Khodakhah, 2003). Importantly, this indicates that SK channels are unlikely to be saturated during spontaneous firing. Therefore, changes in the gating properties of SK channels can in principle influence the firing of Purkinje cells in the absence of defects directly impairing the channel's function.

Given the contribution of P/Q-type and K_{Ca} channels to Purkinje cell excitability, it is not surprising that pharmacologically or genetically manipulating these channels' properties in the cerebellum of mice results in ataxia (Sausbier et al., 2004; Szatanik et al., 2008; Mark et al., 2011). For the output from Purkinje cells to influence movement however, it first has to translate into a change in the activity of DCN neurons, which provide the sole output from the cerebellum.

3.2. Deep cerebellar nuclei

DCN neurons also fire tonically and in the absence of synaptic inputs the coefficient of variation of their interspike interval is also in the order of 0.05, indicating that they do so with great regularity (Raman et al., 2000; Alvina and Khodakhah, 2008). The basal activity of nuclear neurons lies in the middle of their dynamic range and is modulated by inhibitory Purkinje cells input and excitatory mossy and climbing fiber inputs during various cerebellar behaviors (Thach, 1968; Latham and Paul, 1971). Major alterations in the firing of DCN neurons can cause severe motor dysfunction (Shakkottai et al., 2004), indicating the need to evaluate their involvement in the pathomechanism of inherited cerebellar disease.

The ionic factors governing the excitability of nuclear neurons are not as well characterized and are only recently being identified. The tonic activity of nuclear neurons is dependent on calcium influx during an action potential, as demonstrated by the silencing of their firing in the presence of calcium channel blockers (Raman et al., 2000). Recent evidence suggests that the identity of the channel mediating the action potential time-locked calcium current is N-type rather than P/Q- (Alvina and Khodakhah, 2008). Similarly to Purkinje cells, in most DCN neurons these calcium currents do not contribute to the depolarization between spikes and the net current resulting from the activation of voltage-gated calcium channels is outward (Raman et al., 2000), suggesting the coupling of the calcium current to K_{Ca} channels. DCN neurons express both SK and BK channels (Chang et al., 1997; Sausbier et al., 2004; Shakkottai et al., 2004). Block of BK channels results in a slight increase in the firing of DCN neurons, whereas a much larger increase and burst firing accompanies the application of SK channel blockers (Alvina and

Khodakhah, 2008). Thus, current evidence indicates that the major potassium current implicated in maintaining the regularity of DCN neuron firing is mediated by SK channels, the activation of which is dependent on current through channels of the N-type (Alvina and Khodakhah, 2008). Due to the majority of cerebellar research focusing on the properties of cortical neurons, little is known with regard to the effects of Purkinje cells modulation on the firing of DCN neurons. It is clear however that the DCN are not simple relay stations that convey the Purkinje cell output to various brain regions. Evidence for this comes from early demonstrations that when insult to the cerebellum involves damage to the nuclei, the resulting motor symptoms are much more severe and long lasting than when the nuclei are spared (Holmes, 1917).

B. DYSFUNCTION OF THE CEREBELLUM

The symptoms that result from damage to one of the components of the motor control system generally involve problems in either the production of movements or their coordination. These symptoms have traditionally been classified according to the nature of the motor impairment as well as the location of lesions that give rise to them (Donaldson et al., 2012). Thus, extra-pyramidal symptoms result from deficits in the extra-pyramidal brain regions (thalamus, basal ganglia and their connection to and from the motor cortex) and are characterized by the involuntary production of either too little (hypokinetic disorders) or too many (hyperkinetic disorders) movements (Donaldson et al., 2012). These movements can further be distinguished as dystonic, choreic, athetotic, dyskinetic or myoclonic. Dyskinesia refers to any movement that is abnormal and involuntary. Dystonic movements were originally described as slow, sustained, powerful, and non-patterned contortions of the axial and appendicular muscles, with simultaneous

contractions of agonist and antagonist muscles (Lanska, 2010). Myoclonic movements are sudden, shock-like muscular contractions triggered within the central nervous system (Lanska, 2010). On the other hand, cerebellar symptoms result from deficits involving the cerebellum and typically involve an inability to coordinate movements (Holmes, 1922).

1. CEREBELLAR SYMPTOMS

One of the most complete investigation of cerebellar insult in humans was made by Gordon Holmes based on his observations of dozens of gunshot wounds to the cerebellum during the First World War (Holmes, 1917). By meticulously studying the location, extent and outcome of cerebellar injuries, Holmes attempted to analyze the phenomenon of ataxia in its simpler components and concluded that four elementary defects collectively give rise to it. These are 1) dysmetria – abnormalities in the range of movements, 2) deviation from the line of movement – abnormalities in the direction of the movement, 3) dysdiadochokinesia – abnormalities in the timing of reciprocal muscle contractions, 4) asynergia (decomposition of movement) – inability to perform movements requiring the coordination of multiple sets of reciprocal muscles acting at different joints, which gives rise to the decomposition of complex movements into series of simpler ones (Holmes, 1917). It was thus inferred that the cerebellum influences the range or targeting and timing of simple, single-jointed movements as well as their coordination to form complex multi-jointed ones.

Holmes' observations formed the basis of what is today recognized as the cerebellar motor syndrome, which refers to the set of motor symptoms that most often result from deficits involving the cerebellum. In clinical practice, the presence of these symptoms (in the absence of spinal cord and peripheral nerve damage) in an individual

immediately suggests cerebellar dysfunction. The majority of the clinical symptoms associated with a loss of cerebellar function involves some form of ataxia and differ mainly in the muscle groups that are affected. Ataxia is a general term describing deficits of posture, gait, limb, eye movements and speech (Haines and Manto, 2007). Thus, when ataxia involves the arms (limb ataxia), it leads to dysmetria and dysdiadochokinesia. Incoordination of leg muscles results in gait ataxia characterized by clumsy and unsteady ambulation. Deficits in the coordination of axial and trunk muscles lead to abnormal stance and posture. Finally ataxia of bulbar muscles causes slurred speech (dysarthria) an inability to control the volume of speech (speech dysmetria) and defects in oculomotor function such as impaired smooth-pursuit eye movements (Haines and Manto, 2007). Moreover, an inability to coordinate the ensemble of muscles engaged in a given muscle task leads to the decomposition of these movements into their elementary components (decomposition of movement) (Haines and Manto, 2007).

When cerebellar disease is degenerative, the early stage of dysfunction is characterized by a gradual loss of balance, and gait deficits apparent in the inability of the affected individual to perform “tandem gait” (walking with the toes of the back foot touching the heel of the front one) (Haines and Manto, 2007). Gradually the gait widens to compensate for the loss of coordination and balance, and the patient has a tendency to fall or stagger often, as if drunk. Depending on the anatomical distribution of the degeneration, different muscle groups might be affected; ataxia of the limbs can be detected by the inability to bring the tip of the finger to the nose in one smooth motion (finger-to-nose test), as well as movements requiring the rapid and alternate contraction of reciprocal muscles (Haines and Manto, 2007). The ability to legibly write can be lost

entirely and speech may be slurred and incomprehensible. In severe cases the affected individual can be wheelchair-bound, either entirely unable to walk or requiring great assistance to do so (Haines and Manto, 2007). Severe ataxia is clearly extremely disabling to the individual and often associates with a marked decrease in quality of life.

Several scales are used to quantify the impairment ensuing from cerebellar disease in the clinical setting and the ability to conduct therapeutic trials is dependent on their reliability. The International Cooperative Ataxia Rating Scale (ICARS) is standardly used and studies in large-cohorts indicate it has acceptable reliability and validity but is reported to be highly impractical (Schmitz-Hubsch et al., 2006a). This led to the recent development of the Scale for the Assessment and Rating of Ataxia (SARA) (Schmitz-Hubsch et al., 2006b), which is starting to be widely used due to the speed with which it provides equally reliable and valid results (Saute et al., 2012) in a wide range of inherited ataxias (Burk et al., 2009).

At the muscular level, an understanding of the elementary deficits giving rise to ataxia has been studied by performing electromyography (EMG) recordings in humans with cerebellar disease and several characteristic features have been described. In normal subjects, fast flexions of the elbow have a stereotypic pattern of muscle activity, with the contraction of the agonist muscle first, followed by a brief silent period, the contraction of the antagonist and finally that of the agonist one more time (Hallett et al., 1975a). Ataxic patients have defects in the pattern of the phasic burst of muscular activity associated with these movements. The timing of muscular contractions is altered such that the contraction of the antagonist muscle is delayed and its duration is longer, and the

normal pause between the contractions of reciprocal muscles is shorter, resulting in excessive co-contraction of agonist and antagonist muscles (Hallett et al., 1975b).

Ballistic movements are fast single-jointed movements. One way this is accomplished is by asking the subject to hold a manipulandum and to quickly move it to a target by flexing the wrist. Contraction of the reciprocal antagonist muscle of the agonist employed for flexing is required to be able to stop the movement once the target is reached (Berardelli et al., 1996). EMG recordings indicate that in ataxic humans, the onset of antagonist activation is delayed and the rate of rise of antagonist EMG activity is depressed. This is thought to contribute to the subject's inability to accurately reach the target (dysmetria) (Berardelli et al., 1996).

Cerebellar ataxias can originate from a multitude of hereditary and non-hereditary causes. Non-hereditary ataxias arise either from congenital malformations or are acquired as a result of trauma to and infarcts or tumors within the cerebellum, viral infections and nutritional deficiencies (Subramony and Durr, 2012). In addition, likely due to the particular vulnerability of the cerebellum and especially Purkinje cells to environmental damage, exposure to organic solvents or ethanol abuse and other toxic insults are frequent causes of acquired ataxia (Subramony and Durr, 2012).

C. INHERITED DISORDERS OF THE CEREBELLUM

1. ETIOLOGY

Not long after Flourens' "discovery" of the existence of a separate faculty for muscular coordination, Friedreich described the first familial ataxia disorder (Friedreich, 1863). The nature and protein product of mutated genes underlying inherited forms of ataxia is extremely heterogeneous. This diversity however ultimately converges to

manifest as common clinical signs, generally classified as pure cerebellar or extra cerebellar (Manto and Marmolino, 2009). With regard to motor function, pure cerebellar symptoms are characterized by the previously described motor coordination deficits whereas extra-cerebellar symptoms involve various involuntary movements (dyskinesia, dystonia, extra-pyramidal or extra-cerebellar symptoms) (Manto and Marmolino, 2009). Thus, rather than the nature of the symptoms involved, the genetic diversity is reflected in the particular combination of clinical signs in a given patient, the developmental stage at onset, location and extent of the defect, all factors that vary from one affected individual to another (Harding, 1982).

2. CLASSIFICATION

The inherited forms of ataxias are classified into 3 categories according to their mode of inheritance as either autosomal dominant, autosomal recessive or X-linked (Manto, 2010). Autosomal dominant ataxias are divided into 2 groups as spinocerebellar ataxias, which have a slowly progressive disease course and episodic ataxias with episodic occurrence of symptoms superimposed on an otherwise normal or mildly symptomatic baseline phenotype (Manto, 2010).

3. EPISODIC ATAXIAS

In 1946 Parker described the unusual case of 11 patients with “acute bouts” of cerebellar symptoms such as dysarthria and ataxia of gait and limbs (Parker, 1946). This was a bizarre phenotype that could clearly be distinguished from any other form of ataxia by the confinement of cerebellar deficits to episodes characterized by the clear onset and clear resolution of dysfunction (Parker, 1946). In subsequent years, several families and

sporadic cases of this form of episodic ataxia (EA) have been discovered and today 7 different forms, EA1-7, are recognized (Manto, 2010).

The expression of symptoms in EAs is generally triggered by well-characterized stimuli and, although the specific set of triggers differ among the EA subtypes, they all involve some form of physical, psychological or chemical stress (Jen et al., 2007; Manto, 2010). The clinical heterogeneity of EAs in part determines their classification according to the nature of the presenting symptoms as well as the duration of episodes and the stimuli triggering them (Jen et al., 2007; Manto, 2010). Nevertheless there are two overarching features unifying them, namely that they are all channelopathies, caused by mutations in ion channels or transporters, and in all of them the most salient of the episodic symptoms are "cerebellar" in nature (Jen et al., 2007; Manto, 2010). In addition, regardless of the EA subtype, patients can exhibit different combinations of baseline symptoms such as progressive ataxia and nystagmus, dysarthria, dysmetria and impaired smooth-pursuit eye movements between the episodes (Jen et al., 2007; Manto, 2010).

The identification of the mutated genes underlying some of the EAs has led to the expansion of its clinical spectrum to include symptoms such as epilepsy, dystonia, hemiplegic migraine and intermittent coma (Jen et al., 2007).

3.1. Episodic ataxia type 2

The most common form of episodic ataxia is EA2, which is caused by mutations in the *CACNA1A* gene encoding the pore-forming and voltage-sensing α_1 subunit of P/Q-type voltage-gated calcium channels (Ophoff et al., 1996; Denier et al., 1999; Jen et al., 2004; Scoggan et al., 2006). To date nonsense and missense point mutations, splice site and frameshifts have been found in affected individuals (Wan et al., 2011). In addition,

large genomic deletions of *CACNA1A* have recently been reported in several EA2 cases, generating some debate as to whether the deleterious effects always result from a dominant negative effect as previously thought or if haploinsufficiency also plays a role (Wan et al., 2011). Nevertheless, the functional outcome of the mutation is a variable reduction in the P/Q-type calcium current density (Rajakulendran et al., 2010).

In general the baseline phenotype in EA2 is either asymptomatic, or characterized by a slowly progressing ataxia depending on the presence and extent of cerebellar degeneration that can occur in some patients (Jen et al., 2007). The particular stimuli that induce attacks in EA2 are exercise or fatigue, stress, caffeine and ethanol and in response to these stressors, the baseline phenotype is interrupted by episodes of debilitating ataxia, instability and dyskinesia (Baloh and Jen, 2000; Jen et al., 2007; Baloh, 2012). The attacks generally occur frequently, but different patients may experience them daily or only a few times a year and, similarly, the episodes may last minutes or several hours (Jen et al., 2004). Likely due to the underlying genetic heterogeneity, the presence and nature of additional symptoms is highly variable among affected families and can include oculomotor disturbances, episodic weakness, dystonia, migraine, epilepsy and cognitive impairment (Jen et al., 2004).

The mechanism underlying the episodes is unknown, and an effective strategy in their management requires an understanding of the trigger-induced change that causes a transition from mild symptoms to attacks of severe dysfunction. Given the presence of archetypal cerebellar symptoms as well as due to findings from functional imaging studies, the cerebellum is highly implicated in the disease mechanism (Baloh et al.,

1997). The physiological underpinnings of the episodic symptoms are examined in Chapter 2 of this thesis, and the trigger-induction mechanism is the focus in Chapter 3.

3.1.1. Treatments

Acetazolamide (ACTZ) has been the treatment of choice for EA2 for the past decade. Pharmacologically, ACTZ has myriad effects that could in principle underlie its presumed therapeutic mode of action. ACTZ is an inhibitor of carbonic anhydrase, the enzyme that promotes the formation of $CO_2 + H_2O$ from H^+ and HCO_3. Inhibition of this process by ACTZ reduces the levels of bicarbonate in the plasma and alkalizes the intracellular environment. In fact unusually high intracellular pH levels have been observed in EA2 patients (Strupp et al., 2007) and high concentrations of H^+ would be expected to have deleterious consequences on neuronal excitability. Thus, one hypothesis with regard to ACTZ's mode of action is that it normalizes the ionic composition inside the cell by reducing the concentration of H^+.

In addition, recent clinical trials show that 4-aminopyridine (4-AP) provides similar benefits (Strupp et al., 2011). It was recently shown that 4-AP, which activates potassium channels, likely exerts its action by prolonging the action potential and increasing the action potential AHP amplitude (Alvina and Khodakhah, 2010a).

Thus, the use of 4-AP in the treatment of EA2 is likely to increase, especially as an alternative for cases that do not respond to ACTZ or when the side effects of ACTZ are debilitating.

3.1.2. Tottering mouse model

The *tottering* mouse arose as a spontaneous autosomal recessive mutation at the Jackson Laboratory in 1957 and discovered due to its wobbly gait and intermittent

episodes of "motor seizures" (Green and Sidman, 1962). Similarly to humans, its mutation is within the mouse ortholog of the *CACNA1A* gene encoding the α1 subunit of P/Q-type voltage-gated calcium channels (Fletcher et al., 1996). The mutation does not affect the expression levels of the gene product; rather it results in a 40% reduction in the P/Q-type calcium current density (Wakamori et al., 1998). The *tottering*'s syndrome is characterized by 3 different behavioral phenotypes, two of which appear episodically, these are absence seizures, ataxia and paroxysmal dyskinesia. The ataxia is a feature of the "baseline phenotype", in other words it is present all the time, on the other hand the absence seizures and paroxysmal dyskinesia only occur intermittently, in the latter case during discrete episodes or attacks. The dyskinesia episodes can occur spontaneously a few times per day, or in response to the same set of triggers that induce episodes of motor dysfunction in human EA2 patients, namely caffeine, ethanol and stress (Fureman et al., 2002).

3.1.2.1. Absence seizures

In an attempt to define an epileptiform activity associated with the bizarre motor episodes of the *tottering* mouse, initial studies focused on what was originally thought to be intermittent motor seizures. It was through these experiments that investigators unexpectedly discovered abnormal bursts of bilaterally synchronous and symmetrical spike waves over the cerebral hemispheres of the *tottering* mouse at rest (Noebels and Sidman, 1979). This activity comprised about 10% of all EEG activity and it was accompanied by an arrest in movement. This motor arrest and the characteristic EEG pattern bore close resemblance to human absence seizures, which led to the *tottering*

mouse being extensively used as a genetic model of absence epilepsy (Heller et al., 1983).

3.1.2.2. Baseline ataxia

The *tottering*'s baseline motor dysfunction is mild, except for the wide-based stance and an unsteady gait. The *tottering* gait pattern is characterized by a decreased stride and step length as well as an increased gait angle and a closer positioning of the trunk to the ground as compared to wild type (Green and Sidman, 1962; Scholle et al., 2010). The ataxic phenotype can be observed as early as the mice start walking and lead to a poor performance on the rotarod test (Green and Sidman, 1962; Alvina and Khodakhah, 2010b).

3.1.2.3. Paroxysmal dyskinesia

Tottering mice display striking episodes of motor dysfunction, the characteristic symptoms as well as the triggering mechanism of which are examined in chapters 2 and 3. The attacks are highly stereotypical in their duration, symptoms and the manner in which they are induced. These parameters have been numerously quantified by different investigators, and show little variation within and between mice (Shirley et al., 2008). The attacks last approximately 60 to 90 minutes and peak in severity halfway through (Campbell and Hess, 1998; Fureman et al., 2002; Shirley et al., 2008; Alvina and Khodakhah, 2010b). The following is a description of the progression of the *tottering*'s motor symptoms during an episode (Figure 3). The attacks begin on average 10 minutes following the administration of a trigger with a noticeable increase in the baseline ataxia with clumsy placement of the limbs and an unsteady gait that is visually easy to identify. As the attack progresses, the hind limbs begin to display "paddling" or repetitive jerky

movements. These abnormal movements spread rostrally and their severity increases until the limbs retract close to the body and the mouse adopts a hunched, dystonic posture of the trunk with its pelvis extended close to the cage floor. During the peak of the attack, the distal limbs remain extended for several minutes, accompanied by writhing movements of the trunk and sustained flexion of the head and neck, causing the mouse to adopt abnormal postures for several minutes. EMG recordings from agonist and antagonist muscles unambiguously identified the motor symptoms seen during the most severe phase of the attack (approximately 30 minutes after trigger administration) as being dystonic in nature. Following this peak, the symptoms abate within another 30 to 40 minutes, at which point a new attack can be triggered with no discernible refractory period. It has been shown that the attacks are not accompanied by any abnormal EEG discharge (Kaplan et al., 1979; Noebels and Sidman, 1979), ruling out the possibility that they might be motor seizures.

By virtue of its discrete nature, the *tottering* attack is highly amenable to experimental manipulation, making the mouse a particularly useful model. Thus, the induction of dyskinesia (and dystonia) can be controlled through the administration of known doses of caffeine or ethanol and stress paradigms (Fureman et al., 2002). Moreover, the frequency, duration and severity of episodes can be quantified using published scales (Fureman and Hess, 2005; Weisz et al., 2005; Shirley et al., 2008; Alvina and Khodakhah, 2010b). When studying various aspects of the episodic dysfunction, the effect of an experimental manipulation or observation can be compared in the presence and absence of attacks, allowing the mouse to serve as its own control. For these reasons, the attack portion of the *tottering*'s phenotype is extensively studied as

a model of dyskinesia and dystonia (Jinnah and Hess, 2004) and the paroxysmal nature of the symptoms to understand EA2 and episodic neurologic dysfunction in general.

The *tottering*'s symptoms parallel those seen in human EA2 patients, including the induction of episodes by caffeine, ethanol and stress, with the exception of the peak of the mouse's attack being slightly more severe than that of the average seen in humans (Fureman et al., 2002; Jen et al., 2004; Jen et al., 2007; Scholle et al., 2010). Although dystonia does occur as part of the human EA2 phenotype, it is not an invariable feature of all EA2 patients' attacks (Spacey et al., 2005; Cuenca-Leon et al., 2009). Similarly, the occurrence of multiple symptoms and the expression of certain symptoms episodically is a common result of ion channel mutations in humans, making the *tottering* a useful model of episodic neurological dysfunction. Ion channelopathies in fact often share an overlapping clinical spectrum of symptoms with different disorders presenting with variable combinations of such symptoms as migraine, ataxia, epilepsy, dystonia, dyskinesia and hemiplegia (Felix, 2000).

Experiments involving the *tottering* mouse generally focus on one of the three described aspects of its phenotype and it is important to keep the experimental conditions in mind as they determine which aspect of the phenotype, baseline or episodic the findings apply to. The episodic symptoms require experiments to be performed in the awake behaving mouse, since attacks cannot be induced under anesthesia. Therefore, most observations done in the anesthetized or *in vitro* preparations cannot automatically be applied to the paroxysmal aspect of the phenotype. The cerebellum is required for the expression of the episodic symptoms as removing them abolishes the attacks (Neychev et al., 2008). Purkinje cells are specifically implicated because their elimination by the

introduction of the purkinje cell degeneration mutation into the *tottering* background also abolishes the attacks (Campbell et al., 1999). Finally the post-natal, Purkinje cell- specific expression of the *tottering* mutation is sufficient to recapitulate both the baseline as well as the spectrum of the mutant's episodic symptoms (Mark et al., 2011). At the gross morphological level, the cytoarchitecture of the cerebellum and brain stem is not altered in the *tottering* mouse (Green and Sidman, 1962; Noebels and Sidman, 1979; Levitt and Noebels, 1981). There is an increased innervation of the cerebellum and several other brain regions by noradrenergic axons of the locus ceruleus (Levitt and Noebels, 1981). Within the cerebellum, defects at several sites have been hypothesized to underlie the baseline and episodic dysfunction.

P/Q-type voltage-gated calcium channels are expressed throughout the brain; however their proper functioning is especially crucial to cerebellar function where the α_1 subunit is highly expressed (Mintz et al., 1992) and regulate the release of neurotransmitter (Regan, 1991; Usowicz et al., 1992). Impaired synaptic transmission is a prominent hypothesis with regard to EA2 pathomechanism, and is supported by the finding that in vitro electrophysiology studies in patients with genetically characterized EA2 show a reduction in end-plate potential quantal content (Jen et al., 2007). However, likely due to their vital contribution to exocytosis, it is found that the decrease in the P/Q-type calcium current is compensated for by an upregulation of N- and R-type voltage-gated calcium channels at multiple synapses including, in the forebrain (Etheredge et al., 2007), the neuromuscular junction (Kaja et al., 2006) at the climbing and parallel fiber-Purkinje cell synapses (Matsushita et al., 2002). While in this way synaptic transmission at these sites is partially rescued, it is not entirely normal and bi-directional defects in

synaptic transmission were shown to persist at parallel fiber-Purkinje cell synapses (Matsushita et al., 2002). On the other hand, synaptic transmission at the climbing fiber-Purkinje cell synapse is not impaired (Matsushita et al., 2002). Due to the compensatory upregulation of N-type calcium channel expression at granule cell-Purkinje cell synapse, it was found that neurotransmission at this site is much more susceptible to G protein-dependent modulation (Zhou et al., 2003). However the consequences of this finding on the mouse's phenotype, if any, are unclear. At the Purkinje cell-DCN synapse, synaptic transmission is largely spared from the deleterious effect of the mutation. Thus, Purkinje cell inputs are encoded normally in the anesthetized *tottering* (Hoebeek et al., 2005).

As previously described, the activation of P/Q-type calcium channel by an action potential waveform plays a major role in Purkinje cell excitability and the calcium current it mediates is tightly coupled to the activation of SK channels. As a result of this coupling, in Purkinje cells the net P/Q-mediated current is outward and the consequence of pharmacological P/Q channel blockade is an increase in the cell's firing rate followed by bursting and depolarization block (Edgerton and Reinhart, 2003; Womack et al., 2004). In the *tottering* mouse, the P/Q-type calcium current is reduced but not absent, consequently it has been shown that its Purkinje cells are not driven to depolarization block, but fire very irregularly (Wakamori et al., 1998; Hoebeek et al., 2005; Walter et al., 2006). This irregular firing is maintained in the absence of synaptic inputs, indicating that the mutation alters Purkinje cell's intrinsic properties and thus decreases the regularity of their pacemaking (Walter et al., 2006). Given that it is the regularity of this pacemaking that is thought to allow Purkinje cells to reliably encode signals as changes

in the rate and pattern of their firing (Ito, 1984), such decreases in the regularity of pacemaking have deleterious consequences on motor function.

For example although *tottering* Purkinje cells can properly modulate in response to optokinetic stimulation, the mice exhibit reduced gain values during the optokinetic and vestibulo-ocular reflexes (Hoebeek et al., 2005). These compensatory eye movements depend on the ability of floccular Purkinje cells to precisely encode inputs as changes in their firing rate (Hoebeek et al., 2005). In fact the *tottering*'s gain values are indistinguishable from those of flocculectomized wild types and can be rescued by stimulating floccular Purkinje cells to mimic normal simple spike activity patterns (Hoebeek et al., 2005).

Given the possibility that the diminished calcium current ultimately alters the cell's pacemaking due to a reduced activation of SK channels, in a previous study it was hypothesized that increasing the open-probability of SK channels pharmacologically should restore the regularity of Purkinje cell's firing pattern (Walter et al., 2006). Indeed it was found that either perfusing such drugs directly into the cerebellum or orally administering them not only regularizes the firing pattern of *tottering* Purkinje cells *in vitro*, but alleviates its baseline ataxia (Walter et al., 2006; Alvina and Khodakhah, 2010b).

As mentioned, in EA2 various stressors including caffeine, ethanol, exercise and psychological stress trigger attacks of symptoms (Denier et al., 2001). In fact SK channel activators decrease the frequency with which the *tottering* exhibits attacks of dyskinesia as well (Alvina and Khodakhah, 2010b). However, various agents are known to inhibit attacks in the *tottering*. Blockers of L-type calcium channels (Campbell and Hess, 1999),

noradrenergic receptors (Fureman and Hess, 2005) and potassium channels (Weisz et al., 2005) as well as activators of K_{Ca} channels (Alvina and Khodakhah, 2010b) have all been shown to decrease the frequency of attacks in *tottering*. The mechanism by which these compounds prevent attacks remains elusive and in principle, they could do so by acting at any step in the pathway linking stressors to motor symptoms. For this reason, the efficacy of a drug in preventing attacks does not necessarily shed light on the pathophysiology underlying the transient symptoms.

That synaptic transmission plays a major role in the *tottering*'s baseline ataxia is unlikely, however, whether it contributes to the episodic motor symptoms is unknown. In fact the only clues to the triggers' mechanism of action are that the cerebellum is necessary for the expression of the attacks (removing it abolishes them in the *tottering*) (Neychev et al., 2008) and that targeted expression of the mutation in Purkinje cells is sufficient to recapitulate the paroxysmal phenotype (Mark et al., 2011). This indicates that a change in cerebellar output likely contributes to attacks, given that eliminating it occludes their expression, and that rather than various compensatory changes, the diminished calcium current is likely to be implicated at some point of the signaling pathway that ultimately leads to dyskinesia attacks. There are numerous ways triggers could alter cerebellar output by acting either within or outside the cerebellum. These include affecting 1) Purkinje cell or DCN neuron excitability, 2) input to Purkinje cells or DCN neurons, 3) synaptic integration at the level of Purkinje cells or DCN neurons, 4) synaptic transmission at the parallel fiber-Purkinje cell or Purkinje cell – DCN synapses, among others. Essentially, any mechanism that could result in an altered output from the

cerebellum could in principle lead to the expression of dystonic symptoms. One putative mechanism is tested in chapter 3.

4. SPINOCEREBELLAR ATAXIAS

Spinocerebellar ataxias are characterized by slowly progressing ataxia generally accompanied by cerebellar atrophy and variably by spinal cord and sensory deficits (Paulson, 2009).

SCAs are a group of genetically heterogeneous group of disorders that all share the feature of progressive ataxia (Paulson, 2009). To date, about 31 forms of SCA have been recognized, classified according to the combination of specific mutation or mapped locus and clinical findings (Paulson, 2009). For most, the mutations causing the disorder has been identified and include genes coding for ion channels, proteins, kinases and phosphatases or of unknown function (Paulson, 2009). The progressive degeneration that all SCA patients have in common is usually late-onset, variably progressing and generally correlates with the severity of the symptoms. The patterns of atrophy associated with the SCAs are 1) pure cerebellar atrophy, 2) olivopontocerebellar atrophy and 3) a pattern of global brain atrophy (Paulson, 2009; Fratkin and Vig, 2012). The prominent symptoms of the disease are thought to result from damage to the cerebellum and its connections, and similarly to most inherited forms of ataxia, SCA patients exhibit a combination of cerebellar and extracerebellar deficits (Manto, 2005; Manto, 2010; Seidel et al., 2012).

In order to investigate the cerebellar pathophysiology in SCAs, a genetic mouse model mimicking the mutation and phenotype of SCA8 patients was examined. SCA8 results from a CTG trinucleotide expansion repeat in a noncoding RNA of the kelch-like

1 (*klhl1*) gene (Koob et al., 1999; Ikeda et al., 2012). The onset of symptoms is on average in the fourth or fifth decade, but can range anywhere from 18 to 72 years (Ikeda et al., 2012). The SCA8 symptoms are characterized as purely cerebellar and generally limited to ataxia (Ikeda et al., 2012). Although the progression is slow, with the need for mobility aids occurring approximately 20 years following onset, in severely affected individuals atrophy can progress very rapidly and the patient become non-ambulatory by the fourth decade (Day et al., 2000; Ikeda et al., 2012).

The effect of the SCA8 mutation on the klhl-1 protein product is unknown, however both the global and the Purkinje cell-specific deletion of the *klhl1* gene in mice recapitulate the progressive nature of the disease as well as its effect on brain morphology. Homozygous mice develop slowly progressive ataxia, which begins with gait deficits at 12 weeks and can be detected using the rotarod test at 24 weeks (He et al., 2006). Neither cell loss nor any other form of neurodegeneration is detected in the knockout mouse and the only morphological abnormality is a thinner molecular layer (He et al., 2006). The presence of symptoms in the absence of cell loss allowed us to ask whether in certain SCAs the motor deficits might result from cellular malfunctioning rather than neurodegeneration as is generally assumed for SCAs. Unlike in the *tottering*, the mutated gene in SCA8 does not code for an ion channel. This allowed us to determine whether altered cerebellar physiology could account for motor deficits.

5. AUTOSOMAL RECESSIVE ATAXIAS

Autosomal recessive ataxias are in general characterized by degeneration or abnormal development of the cerebellum (Anheim et al., 2012). The two most common forms are Friedreich's ataxia and ataxia-telangiectasia (Anheim et al., 2012). Except for a

few frequently occurring forms, a large number of recessively inherited ataxias arise in small isolated populations or consanguineous families (Manto, 2010). The most frequently occurring additional signs are peripheral neuropathy, mental retardation, chorea, dystonia, oculomotor abnormalities and pyramidal signs (Anheim et al., 2012). Moreover, mutations in the same autosomal recessive ataxia can result in distinct phenotypes especially with regard to age of onset, disease progression and the presence of extracerebellar signs (Anheim et al., 2012).

Dysequilibrium syndrome is a rare form of recessively inherited cerebellar ataxia characterized by variable congenital malformations of the cerebellum, with some affected individuals presenting with almost perfectly formed cerebella and others missing large parts of it (Manto, 2010). Genotyping in affected families has so far revealed mutations in the genes encoding the very low-density lipoprotein (Boycott and Parboosingh, 1993) and the carbonic anhydrase related-protein VIII (CA8 in humans, car8 in mice) (Turkmen et al., 2006). Homozygotes all show severe cerebellar deficits including gait ataxia, dysarthria, dysmetria, dysdiadochokinesia, nystagmus and impaired smooth pursuit eye movements (Turkmen et al., 2009; Kaya et al., 2011; Najmabadi et al., 2011). In addition, problems maintaining balance results in individuals to display quadripedal gait as their primary mode of ambulation (Turkmen et al., 2009). The distinct gait pattern is described as “bear-like” with marked rigidity of the limbs and no visible flexion of leg joints during ambulation (Turkmen et al., 2009; Kaya et al., 2011).

The *waddles* mouse is a spontaneous mutant discovered in the Jackson Laboratory due to its wobbly side-to-side gait and appendicular dystonia (Jiao et al., 2005). Both symptoms are present and stable throughout the mouse’s lifetime (Jinnah and Hess, 2004;

Jiao et al., 2005). Years prior to its identification as part of a human syndrome, genetic analysis mapped the autosomal recessive *waddles* mutation to exon 19 of the *car8* gene (http://mousemutant.jax.org/articles/waddler.html). Expression of *car8* is restricted to the cerebellum with high expression levels in Purkinje cell soma, axon and dendritic arbor, low levels in the molecular and granule cell layers and virtually no expression elsewhere in the brain (Jiao et al., 2005). The car8 protein product has no enzymatic activity and was recently shown to bind to the regulatory subunit of inositol triphosphate receptor-1 and inhibit calcium release from intracellular stores, however the functional consequences of this are unknown (Hirota et al., 2003). In humans cerebellar hypoplasia is present in some but not all patients to varying degrees (Manto, 2010). Interestingly, among individuals of the same family, the severity of ataxia does not correlate with extent of cerebellar malformation (Kaya et al., 2011). No morphological changes have been detected in the *waddles* mouse upon initial examination at Jackson Laboratory, suggesting that changes at the level of cerebellar physiology may underlie its motor deficits (http://mousemutant.jax.org/articles/waddler.html).

6. SYMPTOMS OF INHERITED CEREBELLAR DISEASE

The insight gained by observing cerebellar loss of function has undoubtedly been invaluable in understanding the cerebellum. However, the inference of cerebellar symptomatology based on these same observations is potentially problematic because cerebellar dysfunction that occurs in humans by and large results from inherited conditions that likely induce subtle changes in physiology and never involve such losses of cerebellar function as sustained with tumors and gunshot wounds. In fact clinical evidence indicates that additional motor symptoms can occur with inherited cerebellar

disease. With regard to motor function, inherited cerebellar disorders can involve in addition to ataxia symptoms characterized by the production of excessive, involuntary or abnormal movements. The occurrence of these symptoms is not uncommon, and have been indicated in addition to ataxia, in ataxia-telangiectasia, ataxia-oculomotor apraxia 1 and 2, a large group of spinocerebellar ataxia including SCA1, 2, 3, 6, 7, 12, 14 and 17, 19, 20, 27 and 36, autosomal recessive cerebellar hypoplasia, EA2, ataxia with vitamin E deficiency, cerebrotendinous xanthomatosis, dentatorubropallidoluysian atrophy, ataxia with co-enzyme Q10 deficiency, mitochondrial spinocerebellar ataxia and epilepsy and infantile onset spinocerebellar ataxia (Bodensteiner et al., 1980; Woods and Taylor, 1992; Nikali and Lonnqvist, 1993; Cavalier et al., 1998; Munchau et al., 1999; Modi et al., 2000; O'Hearn et al., 2001; Sethi and Jankovic, 2002; Hatano et al., 2003; Le Ber et al., 2003; Muzaimi et al., 2003; Roubertie et al., 2003; Hagenah et al., 2004; Le Ber et al., 2004; Spacey et al., 2004; Wu et al., 2004; Schelhaas and van de Warrenburg, 2005; Turkmen et al., 2006; Zarubova and Ruzicka, 2006; Mariotti et al., 2007; Roubertie et al., 2008; Alcalay et al., 2009; Cuenca-Leon et al., 2009; van Gaalen et al., 2011; Emmanuele et al., 2012; Hinnell et al., 2012; Ikeda et al., 2012; Shimojima et al., 2012; Storey and Gardner, 2012).

The frequency with which these symptoms co-occur with disease that are presumed to be cerebellar in origin questions the validity of automatically designating them as “extra-cerebellar” symptoms. There are in fact multiple possibilities that would account for this observation. First, unlike lesions, the effect of mutations is global rather than local. Therefore it is entirely possible that a given mutation alters activity in two different brain regions and thus leads to the expression of various symptoms.

Alternatively, a given mutation might cause multiple symptoms by altering the properties of a single brain region. That these symptoms result from deficits outside of the cerebellum is built into the nomenclature, however evidence in the literature questions the validity of this assumption.

In 1914, Dr. Ramsay Hunt published a report titled "*dyssynergia cerebellaris myoclonica* – primary atrophy of the dentate system", in which he describes a large group of patients under his care at Montefiore Home and Hospital (Hunt, 1914). His patients presented with a previously uncharacterized combination of symptoms involving progressive cerebellar ataxia accompanied by myoclonus (sudden and brief involuntary movements caused by muscular contraction that can arise from a number of etiologies) (Hunt, 1914). Interestingly, subsequent autopsies on a subset of these patients confirmed atrophy of the dentate and superior cerebellar peduncle (Hunt, 1921).

In fact since the beginning of the 20th century, the cerebellum has been implicated in dystonia, dyskinesia, athetosis, chorea and myoclonus, all symptoms involving increased muscular activity (Grey, 1916; Urechia et al., 1925; Bostroem and Spatz, 1928; Jakob, 1932; Tsiminakis, 1933; Dow and van Bogaert, 1938; Bürgi, 1945). An interesting example was published by van Bogaert et al. recounting the case of a child presenting with symptoms involving torsion of the trunk and neck, excessive abnormal movements, abnormal and sustained flexions of the limb, dysdiadochokinesia, dysmetria and a "facial ataxia" resulting in slurred speech ("atheto-choreic dystonia with variable dyskinesia and cerebellar signs") (van Bogaert et al., 1951). Upon surgical ablation of a portion of the cerebellar cortex, all the symptoms involving excessive muscle contractions resolved and only certain cerebellar ones remained (an expected outcome of cerebellar ablations) (van

Bogaert et al., 1951). Similar cases involving cerebellar tumors limited to the cerebellum can be found in the literature, remarkably in most such cases the symptoms disappear upon tumor resection (Caress et al., 1996; Krauss et al., 1997). Along the same lines, countless cases detail the occurrence of these symptoms as result of cerebellar infarcts, calcification, lesions and of unknown origin but accompanied by severe ataxia (Nakagaki et al., 2002; Kuoppamaki et al., 2003; Waln and LeDoux, 2010; Usmani et al., 2011; Walsh et al., 2012).

From the fact that movements can be generated in the absence of the cerebellum, it was inferred that 1) the cerebellum does not generate them under normal conditions but also 2) that it is unable to do so. Given that dyskinesia and dystonia symptoms involve increases in muscular activity, this raises an important question: can the cerebellum give rise to muscle contractions under pathological conditions?

D. HYPERKINETIC SYMPTOMS IN CEREBELLAR DISEASE

It is clear from the fact that cerebellectomy does not lead to paralysis that the cerebellum likely does not directly control the ability to generate movements. However, the fact that it could generate muscle contractions has been known for as long as the fact that the cerebellum is involved in motor function.

That electrically stimulating the cerebellum causes muscle contractions was in fact demonstrated by the same experiments that allowed Rolando to determine that the function of the cerebellum was motor in nature (Rolando, 1809). Following the discovery that the motor cortex is “electrically excitable” in 1870 several researchers started investigating whether similar movements could be elicited by stimulating the cerebellar cortex and nuclei. Numerous studies have shown that stimulating the output nuclei causes

muscle contractions in an impressive range of animals including rats, cats, nonhuman primates, birds, alligators, frogs, as well as humans (Clark, 1939a; Goodman, 1958; Goodman and Simpson, 1960; Nashold and Slaughter, 1969; Schultz et al., 1979). The onslaught of data generated by such experiments is now thought to have likely resulted from the antidromic activation of mossy fibers, rather than the action of cerebellar efferents on muscles. However, even early investigators rather quickly took note of this and adjusted their experimental paradigms accordingly. Although the criticism levied against these studies is legitimate, their findings are consistent with experiments in several others in which the same results were demonstrated using alternative techniques.

One example is the mechanical stimulation of the cerebellum, a highly non-physiological technique that involves the insertion of a small needle into the brain to induce motor or behavioral effects. Although it is easy to dismiss mechanical stimulation as rather crude and obviously irrelevant to physiology, there is one significant advantage it has provided early researchers with as compared to electrical stimulations and that is the ability to perform histology in order to ascertain that the damage induced is restricted to the region of interest (Clark, 1939b). The immediate peripheral responses elicited in this way obviously result from the high frequency firing of a group of injured neurons before their death and was thus used as a tool to elicit the firing of a group of neurons without the caveats involved with electrically stimulating them. In 1939 Clark showed that mechanical stimulations of cerebellar lobule VII resulted in seizures involving jerky contractions of the limb muscles, trunk and neck leading to sustained flexion of limbs lasting from a few seconds to 10 minutes (Clark, 1939b; Johnson et al., 1952). The "cerebellar seizure" phenomenon provides further evidence for cerebellar-induced muscle

contractions and although it attracted considerable attention for some time, interest in it gradually weaned likely due to the apparent lack of clinical correlate.

The same effect was also demonstrated multiple times via the application of various pharmacological agents on the surface of the cerebellar cortex. For example, early investigators experimented with strychnine, an antagonist of glycine receptors at the time known as a convulsive agent. In 1926 Miller observed that application of 1 percent strychnine on the surface of the cerebellar cortex resulted in the extension of the head and neck, together with jerky movements of the forelimb, an increase in extensor in muscle tone of the forelimbs and abnormal contractions of trunk muscles resulting in a hunched posture (Miller, 1926). In subsequent experiments the single unit activity of cerebellar cortical neurons was recorded in the presence of locally applied or systemically injected strychnine (Brookhart et al., 1950), and it was found that this led to burst firing of neurons, even when the animal had been curarized prior to strychnine administration (Bremer and Bonnet, 1953). In the presence of strychnine, the firing of cerebellar cortical neurons was described as “outbursts” of high frequency firing punctuated with silences during which the neuron would stop firing, a pattern that continued for one hour after the application of strychnine at which point the firing of the neuron returned to its original pattern (Brookhart et al., 1950). The same pattern of activity was also recorded during mechanical stimulation of the cerebellar cortex and it was in fact noted that the observed burst-pause pattern “forms the pattern of all forms of convulsive activity in cerebellar neurons” (Brookhart et al., 1950).

Studies investigating the outcome of chemically manipulating cerebellar activity have re-emerged in relation to clinical observations implicating the cerebellum in

dyskinesia and dystonia. For example it was recently shown that local injections of AMPA receptor agonists (Fan et al., 2012) and inhibitors of the Na/K-ATPase into the cerebellum result in dystonia (Calderon et al., 2011).

Evidence from ablation experiments indicates that a loss of cerebellar function results in ataxia. Interestingly, as previously mentioned for the *tottering* mouse, in several dystonic mouse models, ablating the cerebellum results in an alleviation of dystonic symptoms (along with the appearance of ataxia, as expected from ablating the cerebellum). In fact, lesions of deep cerebellar nuclei in humans have been found to improve dystonic symptoms as well (Heimburger, 1967; Zervas et al., 1967; Fraioli and Guidetti, 1975). Clearly symptoms involving increased muscular activity do not arise from a loss of cerebellar function but rather from a change in its output. Therefore, that both ataxia and dystonia can occur simultaneously in several inherited disorders as well as their associated animal model is counterintuitive.

Symptoms associated with the involuntary production of movements clearly form a significant portion of what patients suffer from as a result of cerebellar dysfunction. Experimental evidence also indicates that the cerebellum is physically able to produce abnormal muscle contractions. Given the frequent association of dyskinesia and dystonia with inherited ataxias, the cerebellar pathology associated with their expression was examined.

SUMMARY

The overarching aim of this thesis is to examine cerebellar pathophysiology associated with the symptoms that occur as part of inherited cerebellar disorders. Chapter 2 and 3 investigate a physiological basis for cerebellum's debated involvement in the expression of abnormal involuntary movements and examine the paroxysmal nature of these symptoms in EA2. In Chapter 4, whether spinocerebellar ataxias always result from neuronal degeneration was evaluated by examining the physiological underpinnings of SCA8. Chapter 5 investigated whether cerebellar activity is altered in a mutant mouse whose genetic mutations in humans results in cerebellar malformation. Although the mutated genes in episodic ataxia, spinocerebellar ataxia and cerebellar hypoplasia are different and the symptoms involve dyskinesia and dystonia in the former and mild ataxia in the latter, similar aberrant cerebellar output patterns were found to associate with all three syndromes. The final chapter investigated the hypothesis that similar changes in the firing pattern of cerebellar output neurons can give rise to a spectrum of motor dysfunction.

REFERENCES

Afifi AK, Bergman RA (1997) Functional Neuroanatomy: Text and Atlas: McGraw-Hill.

Alcalay R, Wu S, Patel S, Frucht S (2009) Oromandibular dystonia as a complication of cerebrotendinous xanthomatosis. Movement disorders : official journal of the Movement Disorder Society 24:1397-1399.

Allen D, Fakler B, Maylie J, Adelman JP (2007) Organization and regulation of small conductance Ca2+-activated K+ channel multiprotein complexes. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:2369-2376.

Alvina K, Khodakhah K (2008) Selective regulation of spontaneous activity of neurons of the deep cerebellar nuclei by N-type calcium channels in juvenile rats. The Journal of physiology 586:2523-2538.

Alvina K, Khodakhah K (2010a) The therapeutic mode of action of 4-aminopyridine in cerebellar ataxia. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7258-7268.

Alvina K, Khodakhah K (2010b) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Anheim M, Tranchant C, Koenig M (2012) The autosomal recessive cerebellar ataxias. The New England journal of medicine 366:636-646.

Asanuma C, Thach WT, Jones EG (1980) Nucleus interpositus projection to spinal interneurons in monkeys. Brain research 191:245-248.

Asanuma C, Thach WT, Jones EG (1983a) Brainstem and spinal projections of the deep cerebellar nuclei in the monkey, with observations on the brainstem projections of the dorsal column nuclei. Brain research 286:299-322.

Asanuma C, Thach WT, Jones EG (1983b) Distribution of cerebellar terminations and their relation to other afferent terminations in the ventral lateral thalamic region of the monkey. Brain research 286:237-265.

Asanuma H, Fernandez J, Scheibel ME, Scheibel AB (1974) Characteristics of projections from the nucleus ventralis lateralis to the motor cortex in the cats: an anatomical and physiological study. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 20:315-330.

Baloh RW (2012) Episodic ataxias 1 and 2. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:595-602.

Baloh RW, Jen J (2000) Episodic ataxia type 2 and spinocerebellar ataxia type 6.

Baloh RW, Yue Q, Furman JM, Nelson SF (1997) Familial episodic ataxia: clinical heterogeneity in four families linked to chromosome 19p. Annals of neurology 41:8-16.

Bentivoglio M (1982) The organization of the direct cerebellospinal projections. ProgBrain Res 57:279-291.

Berardelli A, Hallett M, Rothwell JC, Agostino R, Manfredi M, Thompson PD, Marsden CD (1996) Single-joint rapid arm movements in normal subjects and in patients with motor disorders. Brain : a journal of neurology 119 (Pt 2):661-674.

Bloom FE, Hoffer BJ, Siggins GR (1971) Studies on norepinephrine-containing afferents to Purkinje cells of art cerebellum. I. Localization of the fibers and their synapses. Brain Res 25:501-521.

Bodensteiner JB, Goldblum RM, Goldman AS (1980) Progressive dystonia masking ataxia in ataxia-telangiectasia. Archives of neurology 37:464-465.

Bostroem A, Spatz H (1928) Bindearmatrophie bei idiopathischer Athetose. Zentralbl f d ges Neurol u Psychiat 48:560-561.

Boycott KM, Parboosingh JS (1993) VLDLR-Associated Cerebellar Hypoplasia. In: GeneReviews (Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, eds). Seattle (WA).

Bremer F, Bonnet V (1953) Action de la strychnine sur les résponses sensorielles et sur les potentiels électriques spotanés de l'écorce cérébelleuse: L'activité convulsive du cervelet. Folia psychiat, neurol et neurochir néerl 56:438-446.

Brookhart JM, Moruzzi G, Snider RS (1950) Spike discharges of single units in the cerebellar cortex. J Neurophysiol 13:465-486.

Bürgi S (1945) Die Bindearm-Chorea im Lichte der experimentellen Forschung. Schweiz med Wchnschr 75:1036-1038.

Burk K, Malzig U, Wolf S, Heck S, Dimitriadis K, Schmitz-Hubsch T, Hering S, Lindig TM, Haug V, Timmann D, Degen I, Kruse B, Dorr JM, Ratzka S, Ivo A, Schols L, Boesch S, Klockgether T, Klopstock T, Schulz JB (2009) Comparison of three clinical rating scales in Friedreich ataxia (FRDA). Movement disorders : official journal of the Movement Disorder Society 24:1779-1784.

Calderon DP, Fremont R, Kraenzlin F, Khodakhah K (2011) The neural substrates of rapid-onset Dystonia-Parkinsonism. Nature neuroscience 14:357-365.

Campbell DB, Hess EJ (1998) Cerebellar circuitry is activated during convulsive episodes in the tottering (tg/tg) mutant mouse. Neuroscience 85:773-783.

Campbell DB, Hess EJ (1999) L-type calcium channels contribute to the tottering mouse dystonic episodes. Molecular pharmacology 55:23-31.

Campbell DB, North JB, Hess EJ (1999) Tottering mouse motor dysfunction is abolished on the Purkinje cell degeneration (pcd) mutant background. Experimental neurology 160:268-278.

Caress JB, Nohria V, Fuchs H, Boustany RM (1996) Torticollis acquired in late infancy due to a cerebellar gangliocytoma. International journal of pediatric otorhinolaryngology 36:39-44.

Cavalier L, Ouahchi K, Kayden HJ, Di Donato S, Reutenauer L, Mandel JL, Koenig M (1998) Ataxia with isolated vitamin E deficiency: heterogeneity of mutations and phenotypic variability in a large number of families. American journal of human genetics 62:301-310.

Chan-Palay V (1977a) In: Cerebellar dentate nucleus. Organization, cytology, and transmitters. Berlin: Springer

Chan-Palay V (1977b) Cerebellar dentate nucleus: Organization, cytology and transmitters. New York: Springer-Verlag.

Chang CP, Dworetzky SI, Wang J, Goldstein ME (1997) Differential expression of the alpha and beta subunits of the large-conductance calcium-activated potassium channel: implication for channel diversity. Brain research Molecular brain research 45:33-40.

Clark SL (1939a) Responses following electrical stimulation of the cerebellar cortex in the normal cat. The Journal of physiology 2:19-35.

Clark SL (1939b) Motor seizures accompanying small cerebellar lesions in cats. J Comp Neurol 71:41-57.

Cohen D, Chambers WW, Sprague JM (1958) Experimental study of the efferent projections from the cerebellar nuclei to the brainstem of the cat. The Journal of comparative neurology 109:233-259.

Cuenca-Leon E, Banchs I, Serra SA, Latorre P, Fernandez-Castillo N, Corominas R, Valverde MA, Volpini V, Fernandez-Fernandez JM, Macaya A, Cormand B (2009) Late-onset episodic ataxia type 2 associated with a novel loss-of-function mutation in the CACNA1A gene. Journal of the neurological sciences 280:10-14.

Day JW, Schut LJ, Moseley ML, Durand AC, Ranum LP (2000) Spinocerebellar ataxia type 8: clinical features in a large family. Neurology 55:649-657.

Deister CA, Chan CS, Surmeier DJ, Wilson CJ (2009) Calcium-activated SK channels influence voltage-gated ion channels to determine the precision of firing in globus pallidus neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:8452-8461.

Denier C, Ducros A, Durr A, Eymard B, Chassande B, Tournier-Lasserve E (2001) Missense CACNA1A mutation causing episodic ataxia type 2. Archives of neurology 58:292-295.

Denier C, Ducros A, Vahedi K, Joutel A, Thierry P, Ritz A, Castelnovo G, Deonna T, Gerard P, Devoize JL, Gayou A, Perrouty B, Soisson T, Autret A, Warter JM, Vighetto A, Van Bogaert P, Alamowitch S, Roullet E, Tournier-Lasserve E (1999) High prevalence of CACNA1A truncations and broader clinical spectrum in episodic ataxia type 2. Neurology 52:1816-1821.

Donaldson I, Marsden CD, Bhatia K (2012) Marsden's Book of Movement Disorders. New York: Oxford Umiversity Press.

Dow RS, van Bogaert L (1938) On complex involuntary movements appearing late following resection of a cerebellar hemisphere. J belge de neurol et de psychiat 38.

Edgerton JR, Reinhart PH (2003) Distinct contributions of small and large conductance Ca2+-activated K+ channels to rat Purkinje neuron function. JPhysiol 548:53-69.

Emmanuele V, Lopez LC, Berardo A, Naini A, Tadesse S, Wen B, D'Agostino E, Solomon M, Dimauro S, Quinzii C, Hirano M (2012) Heterogeneity of Coenzyme Q10 Deficiency: Patient Study and Literature Review. Archives of neurology.

Etheredge JA, Murchison D, Abbott LC, Griffith WH (2007) Functional compensation by other voltage-gated Ca2+ channels in mouse basal forebrain neurons with Ca(V)2.1 mutations. Brain Res 1140:105-119.

Fan X, Hughes KE, Jinnah HA, Hess EJ (2012) Selective and sustained alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor activation in cerebellum induces dystonia in mice. The Journal of pharmacology and experimental therapeutics 340:733-741.

Felix R (2000) Channelopathies: ion channel defects linked to heritable clinical disorders. JMedGenet 37:729-740.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy JD, Jr., Hawkes R, Frankel WN, Copeland NG, Jenkins NA (1996) Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 87:607-617.

Flourens MJP (1824) Researches expérimentales sur le propriétés et fonctions du système nerveux dans les animaux vertébrés. Paris: Cervot.

Fraioli B, Guidetti (1975) Effects of stereotactic lesions of the dentate nucleus of the cerebellum in man. Applied neurophysiology 38:81-90.

Fratkin JD, Vig PJS (2012) Neuropathology of degenerative ataxias: Elsevier.

Friedreich N (1863) Uber degenerative Atrophie der spinalen Hinterstrange. Virchow's Archiv fur Pathologische Anatomie und Physiologie und fur Klinische Medicin 26:391-419.

Fureman BE, Hess EJ (2005) Noradrenergic blockade prevents attacks in a model of episodic dysfunction caused by a channelopathy. Neurobiology of disease 20:227-232.

Fureman BE, Jinnah HA, Hess EJ (2002) Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. Pharmacology, biochemistry, and behavior 73:631-637.

Goodman DC (1958) Cerebellar stimulation in the unanesthetized bullfrog. The Journal of comparative neurology 110:321-335.

Goodman DC, Simpson JT, Jr. (1960) Cerebellar stimulation in the unrestrained and unanesthetized alligator. The Journal of comparative neurology 114:127-135.

Gould BB, Graybiel AM (1976) Afferents to the cerebellar cortex in the cat: evidence for an intrinsic pathway leading from the deep nuclei to the cortex. Brain research 110:601-611.

Green MC, Sidman RL (1962) Tottering--a neuromusclar mutation in the mouse. And its linkage with oligosyndacylism. JHered 53:233-237.

Grey EG (1916) Studies on the Localization of Cerebellar Tumors: The Position of the Head and Suboccipital Discomforts. Annals of surgery 63:129-139.

Hagenah JM, Zuhlke C, Hellenbroich Y, Heide W, Klein C (2004) Focal dystonia as a presenting sign of spinocerebellar ataxia 17. Movement disorders : official journal of the Movement Disorder Society 19:217-220.

Haines DE, Manto MU (2007) Clinical symptoms of cerebellar disease and their interpretation. Cerebellum 6:360-374.

Haines DE, Dietrichs E (2012) The cerebellum - structure and connections. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:3-36.

Hallett M, Shahani BT, Young RR (1975a) EMG analysis of stereotyped voluntary movements in man. Journal of neurology, neurosurgery, and psychiatry 38:1154-1162.

Hallett M, Shahani BT, Young RR (1975b) EMG analysis of patients with cerebellar deficits. Journal of neurology, neurosurgery, and psychiatry 38:1163-1169.

Hallworth NE, Wilson CJ, Bevan MD (2003) Apamin-sensitive small conductance calcium-activated potassium channels, through their selective coupling to voltage-gated calcium channels, are critical determinants of the precision, pace, and pattern of action potential generation in rat subthalamic nucleus neurons in vitro. JNeurosci 23:7525-7542.

Harding AE (1982) The clinical features and classification of the late onset autosomal dominant cerebellar ataxias. A study of 11 families, including descendants of the 'the Drew family of Walworth'. Brain : a journal of neurology 105:1-28.

Hatano T, Okuma Y, Iijima M, Fujishima K, Goto K, Mizuno Y (2003) Cervical dystonia in dentatorubral-pallidoluysian atrophy. Acta neurologica Scandinavica 108:287-289.

Hausser M, Clark BA (1997) Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. Neuron 19:665-678.

He Y, Zu T, Benzow KA, Orr HT, Clark HB, Koob MD (2006) Targeted deletion of a single Sca8 ataxia locus allele in mice causes abnormal gait, progressive loss of motor coordination, and Purkinje cell dendritic deficits. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:9975-9982.

Heimburger RF (1967) Dentatectomy in the treatment of dyskinetic disorders. Confinia neurologica 29:101-106.

Heller AH, Dichter MA, Sidman RL (1983) Anticonvulsant sensitivity of absence seizures in the tottering mutant mouse. Epilepsia 24:25-34.

Hinnell C, Haider S, Delamont S, Clough C, Hadzic N, Samuel M (2012) Dystonia in mitochondrial spinocerebellar ataxia and epilepsy syndrome associated with novel recessive POLG mutations. Movement disorders : official journal of the Movement Disorder Society 27:162-163.

Hirota J, Ando H, Hamada K, Mikoshiba K (2003) Carbonic anhydrase-related protein is a novel binding protein for inositol 1,4,5-trisphosphate receptor type 1. The Biochemical journal 372:435-441.

Hoebeek FE, Stahl JS, van Alphen AM, Schonewille M, Luo C, Rutteman M, van den Maagdenberg AM, Molenaar PC, Goossens HH, Frens MA, De Zeeuw CI (2005) Increased noise level of purkinje cell activities minimizes impact of their modulation during sensorimotor control. Neuron 45:953-965.

Holmes G (1917) The symptoms of acute cerebellar injuries due to gunshot injuries. Brain : a journal of neurology 40:461-535.

Holmes G (1922) Clinical symptoms of cerebellar disease and their interpretation. Lecture I. The Lancet 200:111-115.

Hoshi E, Tremblay L, Feger J, Carras PL, Strick PL (2005) The cerebellum communicates with the basal ganglia. NatNeurosci 8:1491-1493.

Hunt JR (1921) Dyssynergia cerebellaris progressiva. Primary atrophy of the demtate system: um.A contribution to the pathology and symptomatology of the cerebell. Brain : a journal of neurology 44:490-538.

Hunt R (1914) Dyssynergia cerebellaris progressiva - a chronic progressive form of cerebellar tremor. Brain : a journal of neurology 37:247-268.

Ikeda Y, Ranum LP, Day JW (2012) Clinical and genetic features of spinocerebellar ataxia type 8. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:493-505.

Ito M (1984) The Cerebellum and Neural Control. New York: Raven.

Ito M, Yoshida M, Obata K, Kawai N, Udo M (1970) Inhibitory control of intracerebellar nuclei by the purkinje cell axons. ExpBrain Res 10:64-80.

Jakob A (1932) Zur Frage der nosologischen und lokalisatorischen Auffassung der torsiondystonischen Krankhetiserscheinungen. Deutsche Ztschr f Nervenh 124:148.

Jen J, Kim GW, Baloh RW (2004) Clinical spectrum of episodic ataxia type 2. Neurology 62:17-22.

Jen JC, Graves TD, Hess EJ, Hanna MG, Griggs RC, Baloh RW, investigators C (2007) Primary episodic ataxias: diagnosis, pathogenesis and treatment. Brain : a journal of neurology 130:2484-2493.

Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, Gu W (2005) Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. Genetics 171:1239-1246.

Jinnah HA, Hess EJ (2004) Animal Models of Movement Disorders. San Diego: Elsevier.

Johnson HC, Browne KM, Markham JW (1952) Experimental cerebellar seizures. Arch NeuroPsych 67:473-482.

Kaja S, Van de Ven RC, Ferrari MD, Frants RR, Van den Maagdenberg AM, Plomp JJ (2006) Compensatory contribution of Cav2.3 channels to acetylcholine release at the neuromuscular junction of tottering mice. Journal of neurophysiology 95:2698-2704.

Kaplan BJ, Seyfried TN, Glaser GH (1979) Spontaneous polyspike discharges in an epileptic mutant mouse (tottering). Experimental neurology 66:577-586.

Kaya N, Aldhalaan H, Al-Younes B, Colak D, Shuaib T, Al-Mohaileb F, Al-Sugair A, Nester M, Al-Yamani S, Al-Bakheet A, Al-Hashmi N, Al-Sayed M, Meyer B, Jungbluth H, Al-Owain M (2011) Phenotypical spectrum of cerebellar ataxia associated with a novel mutation in the CA8 gene, encoding carbonic anhydrase (CA) VIII. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 156B:826-834.

Koob MD, Moseley ML, Schut LJ, Benzow KA, Bird TD, Day JW, Ranum LP (1999) An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8). Nature genetics 21:379-384.

Krauss JK, Seeger W, Jankovic J (1997) Cervical dystonia associated with tumors of the posterior fossa. Mov Disord 12:443-447.

Kuoppamaki M, Giunti P, Quinn N, Wood NW, Bhatia KP (2003) Slowly progressive cerebellar ataxia and cervical dystonia: clinical presentation of a new form of spinocerebellar ataxia? Movement disorders : official journal of the Movement Disorder Society 18:200-206.

Lanska DJ (2010) Chapter 33: the history of movement disorders. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 95:501-546.

Latham A, Paul DH (1971) Spontaneous activity of cerebellar Purkinje cells and their responses to impulses in climbing fibres. The Journal of physiology 213:135-156.

Le Ber I, Moreira MC, Rivaud-Pechoux S, Chamayou C, Ochsner F, Kuntzer T, Tardieu M, Said G, Habert MO, Demarquay G, Tannier C, Beis JM, Brice A, Koenig M, Durr A

(2003) Cerebellar ataxia with oculomotor apraxia type 1: clinical and genetic studies. Brain : a journal of neurology 126:2761-2772.

Le Ber I, Bouslam N, Rivaud-Pechoux S, Guimaraes J, Benomar A, Chamayou C, Goizet C, Moreira MC, Klur S, Yahyaoui M, Agid Y, Koenig M, Stevanin G, Brice A, Durr A (2004) Frequency and phenotypic spectrum of ataxia with oculomotor apraxia 2: a clinical and genetic study in 18 patients. Brain : a journal of neurology 127:759-767.

Levitt P, Noebels JL (1981) Mutant mouse tottering: selective increase of locus ceruleus axons in a defined single-locus mutation. ProcNatlAcadSciUSA 78:4630-4634.

Lisberger SG, Fuchs AF (1978) Role of primate flocculus during rapid behavioral modification of vestibuloocular reflex. I. Purkinje cell activity during visually guided horizontal smooth-pursuit eye movements and passive head rotation. JNeurophysiol 41:733-763.

Luciani L (1891) Il cervelletto: nuovi studi di fisiologia normale e patologica. Firenze: Le Monnier.

Manto M (2005) The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum 4:2-6.

Manto M, Marmolino D (2009) Cerebellar ataxias. Current opinion in neurology 22:419-429.

Manto MU (2010) Cerebellar Disorders. New York: Cambridge University Press.

Mariotti C, Alpini D, Fancellu R, Soliveri P, Grisoli M, Ravaglia S, Lovati C, Fetoni V, Giaccone G, Castucci A, Taroni F, Gellera C, Di Donato S (2007) Spinocerebellar ataxia type 17 (SCA17): oculomotor phenotype and clinical characterization of 15 Italian patients. Journal of neurology 254:1538-1546.

Mark MD, Maejima T, Kuckelsberg D, Yoo JW, Hyde RA, Shah V, Gutierrez D, Moreno RL, Kruse W, Noebels JL, Herlitze S (2011) Delayed postnatal loss of P/Q-type calcium channels recapitulates the absence epilepsy, dyskinesia, and ataxia phenotypes of genomic Cacna1a mutations. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4311-4326.

Matsushita K, Wakamori M, Rhyu IJ, Arii T, Oda S, Mori Y, Imoto K (2002) Bidirectional alterations in cerebellar synaptic transmission of tottering and rolling Ca2+ channel mutant mice. JNeurosci 22:4388-4398.

Matsushita M, Iwahori N (1971a) Structural organization of the interpositus and the dentate nuclei. Brain Res 35:17-36.

Matsushita M, Iwahori N (1971b) Structural organization of the fastigial nucleus. I. Dendrites and axonal pathways. Brain research 25:597-610.

Matsushita M, Hosoya Y (1978) The location of spinal projection neurons in the cerebellar nuclei (cerebellospinal tract neurons) of the cat. A study with the horseradish peroxidase technique. Brain research 142:237-248.

Miller FR (1926) The action of strychnine on the cerebellar cortex. Nature 117:486-487.

Mintz IM, Adams ME, Bean BP (1992) P-type calcium channels in rat central and peripheral neurons. Neuron 9:85-95.

Modi G, Modi M, Martinus I, Rodda J, Saffer D (2000) The clinical and genetic characteristics of spinocerebellar ataxia type 7 (SCA 7) in three Black South African families. Acta neurologica Scandinavica 101:177-182.

Mullen RJ, Eicher EM, Sidman RL (1976) Purkinje cell degeneration, a new neurological mutation in the mouse. ProcNatlAcadSciUSA 73:208-212.

Munchau A, Dressler D, Bhatia KP, Vogel P, Zuhlke C (1999) Machado-Joseph disease presenting as severe generalised dystonia in a German patient. Journal of neurology 246:840-842.

Muzaimi MB, Wiles CM, Robertson NP, Ravine D, Compston DA (2003) Task specific focal dystonia: a presentation of spinocerebellar ataxia type 6. Journal of neurology, neurosurgery, and psychiatry 74:1444-1445.

Najmabadi H et al. (2011) Deep sequencing reveals 50 novel genes for recessive cognitive disorders. Nature 478:57-63.

Nakagaki H, Furuya H, Miyoshi Y, Murai H, Araki T, Ohyagi Y, Yamada T, Sasaki M, Tobimatsu S, Kira J (2002) [A case of cerebellar ataxia showing severe dystonia masquerading as myoclonic jerky movements on arm extension]. Rinsho Shinkeigaku 42:7-12.

Nashold BS, Jr., Slaughter DG (1969) Effects of stimulating or destroying the deep cerebellar regions in man. Journal of neurosurgery 31:172-186.

Neychev VK, Fan X, Mitev VI, Hess EJ, Jinnah HA (2008) The basal ganglia and cerebellum interact in the expression of dystonic movement. Brain : a journal of neurology 131:2499-2509.

Nikali K, Lonnqvist T (1993) Infantile-Onset Spinocerebellar Ataxia. In: GeneReviews (Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, eds). Seattle (WA).

Noebels JL, Sidman RL (1979) Inherited epilepsy: spike-wave and focal motor seizures in the mutant mouse tottering. Science 204:1334-1336.

O'Hearn E, Holmes SE, Calvert PC, Ross CA, Margolis RL (2001) SCA-12: Tremor with cerebellar and cortical atrophy is associated with a CAG repeat expansion. Neurology 56:299-303.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. Cell 87:543-552.

Palay SL, Chan-Palay V (1974) Cerebellar Cortex: Cytology and Organization: Springer.

Palkovits M, Magyar P, Szentagothai J (1971) Quantitative histological analysis of the cerebellar cortex in the cat. I. Number and arrangement in space of the Purkinje cells. Brain research 32:1-13.

Parker HL (1946) Periodic ataxia. Collected Papers Mayo Clin & Mayo Found 38:642-645.

Paulson HL (2009) The spinocerebellar ataxias. Journal of neuro-ophthalmology : the official journal of the North American Neuro-Ophthalmology Society 29:227-237.

Pedarzani P, McCutcheon JE, Rogge G, Jensen BS, Christophersen P, Hougaard C, Strobaek D, Stocker M (2005) Specific enhancement of SK channel activity selectively potentiates the afterhyperpolarizing current I(AHP) and modulates the firing properties of hippocampal pyramidal neurons. The Journal of biological chemistry 280:41404-41411.

Pietrobon D (2002) Calcium channels and channelopathies of the central nervous system. Molecular neurobiology 25:31-50.

Rajakulendran S, Graves TD, Labrum RW, Kotzadimitriou D, Eunson L, Davis MB, Davies R, Wood NW, Kullmann DM, Hanna MG, Schorge S (2010) Genetic and functional characterisation of the P/Q calcium channel in episodic ataxia with epilepsy. JPhysiol 588:1905-1913.

Raman IM, Bean BP (1999a) Properties of sodium currents and action potential firing in isolated cerebellar Purkinje neurons. AnnNYAcadSci 868:93-96.

Raman IM, Bean BP (1999b) Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. JNeurosci 19:1663-1674.

Raman IM, Gustafson AE, Padgett D (2000) Ionic currents and spontaneous firing in neurons isolated from the cerebellar nuclei. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:9004-9016.

Regan LJ (1991) Voltage-dependent calcium currents in Purkinje cells from rat cerebellar vermis. JNeurosci 11:2259-2269.

Rolando L (1809) Saggio sopra: La vera Struttura del Cerevello Dell'Uomo e Degl'Animala. Sassari, Italy.

Roubertie A, Biolsi B, Rivier F, Humbertclaude V, Cheminal R, Echenne B (2003) Ataxia with vitamin E deficiency and severe dystonia: report of a case. Brain & development 25:442-445.

Roubertie A, Echenne B, Leydet J, Soete S, Krams B, Rivier F, Riant F, Tournier-Lasserve E (2008) Benign paroxysmal tonic upgaze, benign paroxysmal torticollis, episodic ataxia and CACNA1A mutation in a family. Journal of neurology 255:1600-1602.

Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF, Ruth P (2004) Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. ProcNatlAcadSciUSA 101:9474-9478.

Saute JA, Donis KC, Serrano-Munuera C, Genis D, Ramirez LT, Mazzetti P, Perez LV, Latorre P, Sequeiros J, Matilla-Duenas A, Jardim LB, On behalf of the Iberoamerican Multidisciplinary Network for the Study of Movement Disorders Study G (2012) Ataxia Rating Scales-Psychometric Profiles, Natural History and Their Application in Clinical Trials. Cerebellum 11:488-504.

Schelhaas HJ, van de Warrenburg BP (2005) Clinical, psychological, and genetic characteristics of spinocerebellar ataxia type 19 (SCA19). Cerebellum 4:51-54.

Schmitz-Hubsch T et al. (2006a) Reliability and validity of the International Cooperative Ataxia Rating Scale: a study in 156 spinocerebellar ataxia patients. Movement disorders : official journal of the Movement Disorder Society 21:699-704.

Schmitz-Hubsch T et al. (2006b) Scale for the assessment and rating of ataxia: development of a new clinical scale. Neurology 66:1717-1720.

Scholle HC, Jinnah HA, Arnold D, Biedermann FH, Faenger B, Grassme R, Hess EJ, Schumann NP (2010) Kinematic and electromyographic tools for characterizing movement disorders in mice. Movement disorders : official journal of the Movement Disorder Society 25:265-274.

Schultz W, Montgomery EB, Jr., Marini R (1979) Proximal limb movements in response to microstimulation of primate dentate and interpositus nuclei mediated by brain-stem structures. Brain : a journal of neurology 102:127-146.

Scoggan KA, Friedman JH, Bulman DE (2006) CACNA1A mutation in a EA-2 patient responsive to acetazolamide and valproic acid. CanJNeurolSci 33:68-72.

Seidel K, Siswanto S, Brunt ER, den Dunnen W, Korf HW, Rub U (2012) Brain pathology of spinocerebellar ataxias. Acta neuropathologica 124:1-21.

Sethi KD, Jankovic J (2002) Dystonia in spinocerebellar ataxia type 6. Movement disorders : official journal of the Movement Disorder Society 17:150-153.

Shakkottai VG, do Carmo Costa M, Dell'Orco JM, Sankaranarayanan A, Wulff H, Paulson HL (2011) Early changes in cerebellar physiology accompany motor dysfunction in the polyglutamine disease spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:13002-13014.

Shakkottai VG, Chou CH, Oddo S, Sailer CA, Knaus HG, Gutman GA, Barish ME, LaFerla FM, Chandy KG (2004) Enhanced neuronal excitability in the absence of neurodegeneration induces cerebellar ataxia. The Journal of clinical investigation 113:582-590.

Shimojima K, Okumura A, Natsume J, Aiba K, Kurahashi H, Kubota T, Yokochi K, Yamamoto T (2012) Spinocerebellar ataxias type 27 derived from a disruption of the fibroblast growth factor 14 gene with mimicking phenotype of paroxysmal non-kinesigenic dyskinesia. Brain & development 34:230-233.

Shirley TL, Rao LM, Hess EJ, Jinnah HA (2008) Paroxysmal dyskinesias in mice. Movement disorders : official journal of the Movement Disorder Society 23:259-264.

Spacey SD, Materek LA, Szczygielski BI, Bird TD (2005) Two novel CACNA1A gene mutations associated with episodic ataxia type 2 and interictal dystonia. Archives of neurology 62:314-316.

Spacey SD, Hildebrand ME, Materek LA, Bird TD, Snutch TP (2004) Functional implications of a novel EA2 mutation in the P/Q-type calcium channel. Annals of neurology 56:213-220.

Storey E, Gardner RJ (2012) Spinocerebellar ataxia type 20. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:567-573.

Strupp M, Zwergal A, Brandt T (2007) Episodic ataxia type 2. Neurotherapeutics 4:267-273.

Strupp M, Kalla R, Claassen J, Adrion C, Mansmann U, Klopstock T, Freilinger T, Neugebauer H, Spiegel R, Dichgans M, Lehmann-Horn F, Jurkat-Rott K, Brandt T, Jen JC, Jahn K (2011) A randomized trial of 4-aminopyridine in EA2 and related familial episodic ataxias. Neurology 77:269-275.

Subramony SH, Durr A (2012) Ataxia disorders: Elsevier.

Szatanik M, Vibert N, Vassias I, Guenet JL, Eugene D, de Waele C, Jaubert J (2008) Behavioral effects of a deletion in Kcnn2, the gene encoding the SK2 subunit of small-conductance Ca2+-activated K+ channels. Neurogenetics 9:237-248.

Thach WT (1968) Discharge of Purkinje and cerebellar nuclear neurons during rapidly alternating arm movements in the monkey. Journal of neurophysiology 31:785-797.

Thach WT (1970) Discharge of cerebellar neurons related to two maintained postures and two prompt movements. II. Purkinje cell output and input. JNeurophysiol 33:537-547.

Tsiminakis Y (1933) Zur Lokalisation der Hemichorea. Arb a d neurol Inst a d Wien Univ, 35:57-75.

Turkmen S, Demirhan O, Hoffmann K, Diers A, Zimmer C, Sperling K, Mundlos S (2006) Cerebellar hypoplasia and quadrupedal locomotion in humans as a recessive trait mapping to chromosome 17p. Journal of medical genetics 43:461-464.

Turkmen S, Guo G, Garshasbi M, Hoffmann K, Alshalah AJ, Mischung C, Kuss A, Humphrey N, Mundlos S, Robinson PN (2009) CA8 mutations cause a novel syndrome characterized by ataxia and mild mental retardation with predisposition to quadrupedal gait. PLoS genetics 5:e1000487.

Urechia CI, Mihalescu S, Elekes N (1925) Contribution anatomo-clinique a l'étude de la dystonie lenticulaire (spasme de torsion). Rev neurol 32:177.

Usmani N, Bedi GS, Sengun C, Pandey A, Singer C (2011) Late onset of cervical dystonia in a 39-year-old patient following cerebellar hemorrhage. Journal of neurology 258:149-151.

Usowicz MM, Sugimori M, Cherksey B, Llinas R (1992) P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. Neuron 9:1185-1199.

van Bogaert L, de Ajuriaguerra MDJ, H.; H, R. T (1951) D'un syndrome dégénératif du groupe de la dystonia à début cérébelleux correspondent à une abiotrophie spino-striée avec dégérescence du système olfactic. Monatschr f Psychiat u Neurol 121:143-162.

van Gaalen J, Giunti P, van de Warrenburg BP (2011) Movement disorders in spinocerebellar ataxias. Movement disorders : official journal of the Movement Disorder Society 26:792-800.

Vergara C, Latorre R, Marrion NV, Adelman JP (1998) Calcium-activated potassium channels. CurrOpinNeurobiol 8:321-329.

Wakamori M, Yamazaki K, Matsunodaira H, Teramoto T, Tanaka I, Niidome T, Sawada K, Nishizawa Y, Sekiguchi N, Mori E, Mori Y, Imoto K (1998) Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. The Journal of biological chemistry 273:34857-34867.

Waln O, LeDoux MS (2010) Delayed-onset oromandibular dystonia after a cerebellar hemorrhagic stroke. Parkinsonism & related disorders 16:623-625.

Walsh RA, Gillivan-Murphy P, Murphy C, Colreavy M, O'Rourke K, Lynch T (2012) Bilateral cerebellar stroke presenting with acute dysphonia and late palatal tremor. Movement disorders : official journal of the Movement Disorder Society 27:346-348.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. Nature neuroscience 9:389-397.

Wan J, Mamsa H, Johnston JL, Spriggs EL, Singer HS, Zee DS, Al-Bayati AR, Baloh RW, Jen JC, Investigators C (2011) Large Genomic Deletions in CACNA1A Cause Episodic Ataxia Type 2. Frontiers in neurology 2:51.

Weisz CJ, Raike RS, Soria-Jasso LE, Hess EJ (2005) Potassium channel blockers inhibit the triggers of attacks in the calcium channel mouse mutant tottering. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4141-4145.

Womack MD, Khodakhah K (2002) Characterization of large conductance Ca2+-activated K+ channels in cerebellar Purkinje neurons. The European journal of neuroscience 16:1214-1222.

Womack MD, Khodakhah K (2003) Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar Purkinje neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:2600-2607.

Womack MD, Chevez C, Khodakhah K (2004) Calcium-activated potassium channels are selectively coupled to P/Q-type calcium channels in cerebellar Purkinje neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:8818-8822.

Womack MD, Hoang C, Khodakhah K (2009) Large conductance calcium-activated potassium channels affect both spontaneous firing and intracellular calcium concentration in cerebellar Purkinje neurons. Neuroscience 162:989-1000.

Woods CG, Taylor AM (1992) Ataxia telangiectasia in the British Isles: the clinical and laboratory features of 70 affected individuals. The Quarterly journal of medicine 82:169-179.

Wu YR, Lee-Chen GJ, Lang AE, Chen CM, Lin HY, Chen ST (2004) Dystonia as a presenting sign of spinocerebellar ataxia type 1. Movement disorders : official journal of the Movement Disorder Society 19:586-587.

Zarubova K, Ruzicka E (2006) Cervical dystonia in spinocerebellar ataxia type 2. Movement disorders : official journal of the Movement Disorder Society 21:1295-1296.

Zervas NT, Horner FA, Pickren KS (1967) The treatment of dyskinesia by stereotxic dentatectomy. Confinia neurologica 29:93-100.

Zhou YD, Turner TJ, Dunlap K (2003) Enhanced G protein-dependent modulation of excitatory synaptic transmission in the cerebellum of the Ca2+ channel-mutant mouse, tottering. The Journal of physiology 547:497-507.

FIGURES

FIGURE 1

Simplified schematic of the major components in the motor control system.

The components of the motor system are interconnected. The two major motor control loops are formed by the corticospinal and brainstem descending pathways (black lines). The thalamus and basal ganglia form sub-loops within the two major loops (black circles). The cerebellum receives input from all the major components in the motor control system (grey lines), and cerebellar efferents make mono-, di- or poly-synaptic connections with all the major brain regions involved in the control of movement (red lines). In addition, the cerebellum forms a monosynaptic connection with the spinal cord.

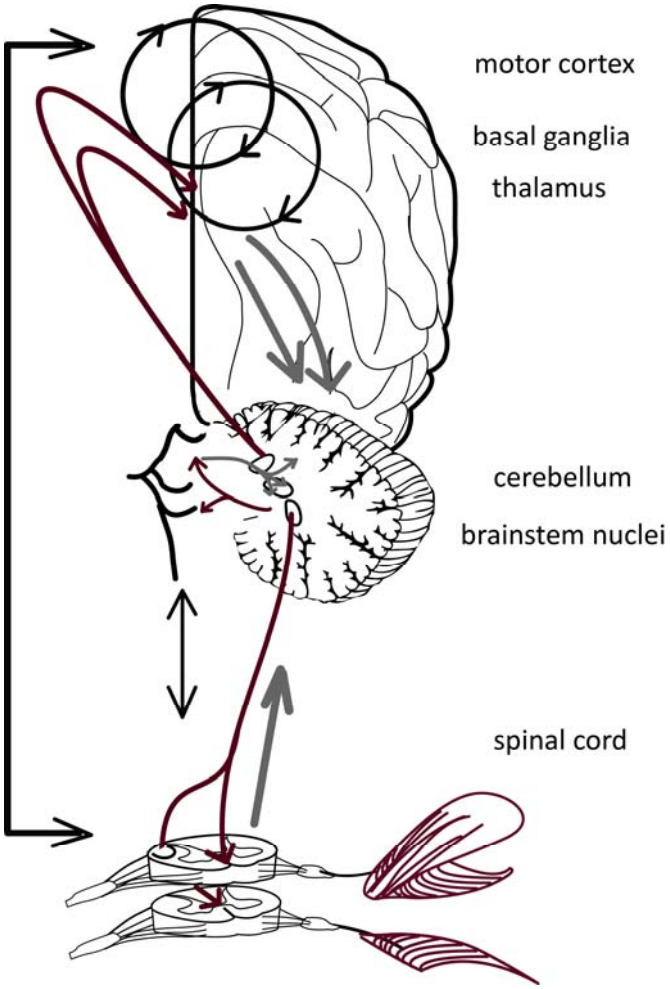

motor cortex
basal ganglia
thalamus
cerebellum
brainstem nuclei
spinal cord

FIGURE 2

Basic cerebellar circuitry.

Excitatory and inhibitory connections in the cerebellar cortex and deep cerebellar nuclei.

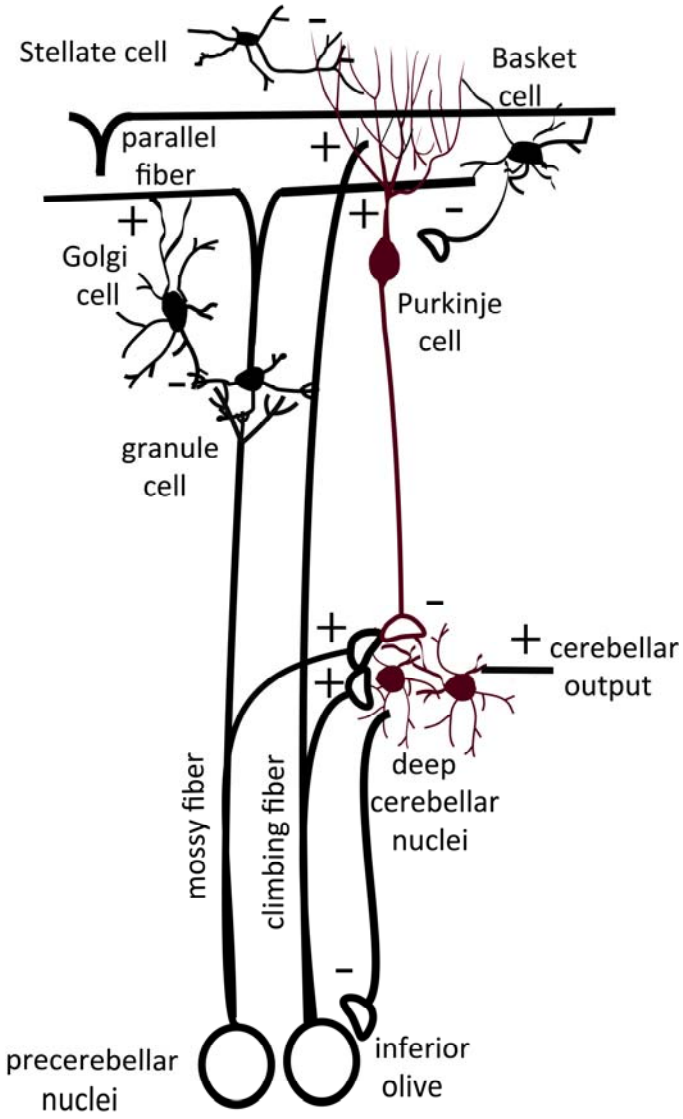
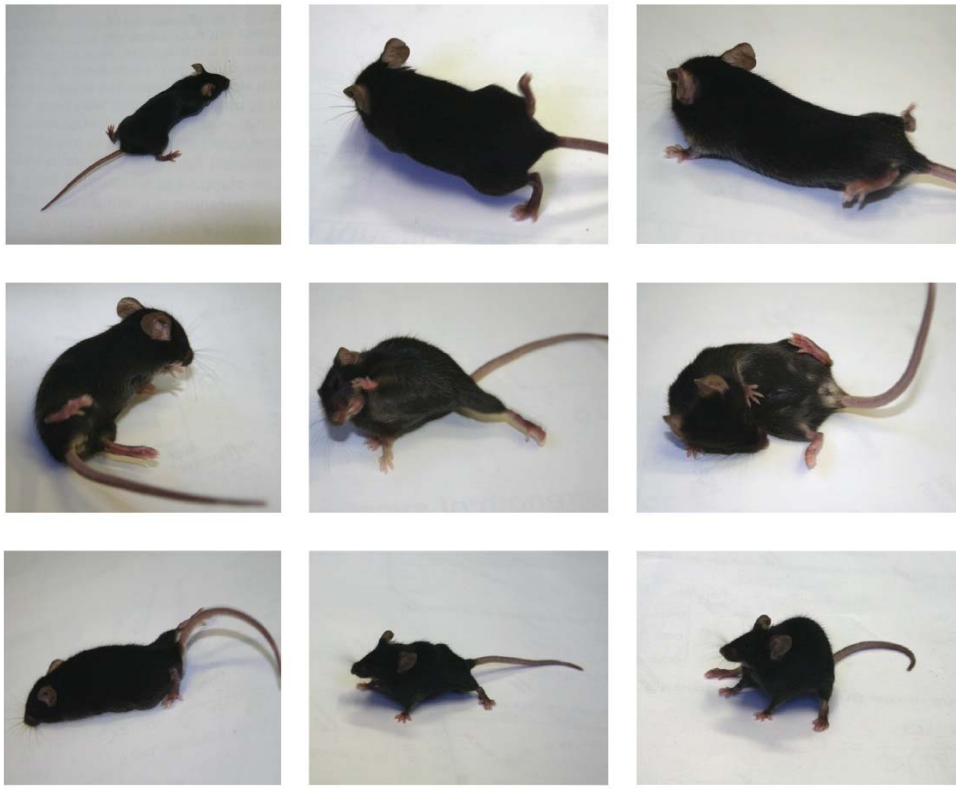

Stellate cell
Basket cell
parallel fiber
Golgi cell
Purkinje cell
granule cell
cerebellar output
deep cerebellar nuclei
mossy fiber
climbing fiber
precerebellar nuclei
inferior olive

FIGURE 3

Stages of the *tottering* mutant's dyskinesia attacks.

The *tottering* mutant displays a characteristic set of motor symptoms during its dyskinesia attacks. The symptoms progress in severity from ataxia to dyskinesia and finally dystonia during the peak of the attack. The pictures were taken at 10 minute intervals.



CHAPTER II:

A CONTINUUM IN ABERRANT CEREBELLAR OUTPUT PATTERNS CAN CAUSE BOTH LOSS OF COORDINATION AND INVOLUNTARY MUSCLE CONTRACTIONS[1]

Esra Tara, Christopher Chen[2], Ellen J. Hess and Kamran Khodakhah

[1] This manuscript is in review at Nature Neuroscience.
[2] Christopher Chen performed the optogenetic experiments.

ABSTRACT

While the best appreciated symptom of cerebellar dysfunction is ataxia, a loss of motor coordination, mounting evidence also implicates the cerebellum in a host of motor disorders such as dyskinesia and dystonia which manifest as involuntary muscle contractions. We used the spectrum of motor dysfunction displayed by the *tottering* mouse, an animal model of episodic ataxia type 2, to delineate the cerebellar output patterns that are associated with these diverse symptoms. Here we show that the severity of the motor symptoms is correlated with the extent of Purkinje cell erratic activity and burst firing, and the consequent aberrant output from the cerebellum. Our findings support the hypothesis that ataxia is caused by the diminished information content of the cerebellar output signals, whereas dystonia and dyskinesia result from bursting activity in the output nuclei acting as erroneous command signals to cause aberrant muscle contractions.

INTRODUCTION

While the cerebellum has been implicated in disorders as diverse as autism (Fatemi et al., 2012), migraine (Russell and Ducros, 2011), schizophrenia (Ho et al., 2004) and dyslexia (Nicolson et al., 2001), its most studied aspects are its role in motor coordination and its dysfunction in ataxia (Ito, 1984). Lesion studies in humans and evidence from patients with progressive degeneration of cerebellar neurons demonstrate that the absence of cerebellar information results in uncoordinated movements and ataxia, and suggest that in healthy individuals cerebellar output ensures the proper timing and coordination of muscles (Ito, 1984). Century old experiments in animals have substantiated this premise and have shown that complete loss of cerebellar function achieved by cerebellectomy leads to severe ataxia (Flourens, 1824). The observation that humans and animals can move even in the complete absence of the cerebellum has driven the widely accepted premise that the cerebellum is not required for, nor does it initiate, muscle contractions.

Yet growing clinical evidence in patients suggests cerebellar involvement in the pathogenesis of a host of motor symptoms associated with increased involuntary muscular activity ranging from sporadic muscle contractions in some forms of dyskinesia, to the sustained co-contractions of agonist and antagonist muscle pairs - a characteristic of dystonia (Heimburger, 1967; LeDoux and Brady, 2003; Le Ber et al., 2006; Sadnicka et al., 2012). Indeed, in several hereditary ataxias a patient's symptoms can at different times manifest as ataxia, dystonia or dyskinesia (Fletcher et al., 1988; Lees, 1990; Woods and Taylor, 1992; Munchau et al., 1999; Modi et al., 2000; Schols et al., 2000; O'Hearn et al., 2001; Sethi and Jankovic, 2002; Muzaimi et al., 2003; Hagenah

et al., 2004; Wu et al., 2004; Zarubova and Ruzicka, 2006; Mariotti et al., 2007). Corroborating a cerebellar role in these disorders, in a few patients surgical lesions of the cerebellar output nuclei have been shown to improve dystonia (Heimburger, 1967; Zervas et al., 1967; Fraioli and Guidetti, 1975) and removal of the cerebellum in the genetically dystonic rat and mouse models of paroxysmal dyskinesia alleviates their dystonia and dyskinesia leaving pure ataxia (LeDoux et al., 1993; Devanagondi et al., 2007; Neychev et al., 2008). At the present there is little information on the nature of aberrant cerebellar outputs that cause each of these symptoms. To address this question, we examined how cerebellar output is affected during a host of diverse motor symptoms in a well-established animal model of episodic ataxia type 2 (EA2), the *tottering* mouse (Pietrobon, 2002; Weisz et al., 2005).

EA2 is associated with mutations in the *CACNA1A* gene which encodes the $Ca_V2.1\alpha_1$ subunit of P/Q-type voltage-gated calcium channel (Ophoff et al., 1996). The consequence of these mutations in EA2 patients and *tottering* mice is a reduction in the P/Q-type calcium current density in cerebellar Purkinje cells and a moderately ataxic baseline phenotype (Wakamori et al., 1998; Rajakulendran et al., 2010a). In response to a diverse set of stressors such as caffeine, ethanol and stress, the mild baseline ataxia in patients is transformed into episodes of debilitating dyskinesia, instability and severe ataxia (Ptacek, 1997; Jen et al., 2004). In some EA2 patients the symptoms also include paroxysmal dyskinesia as well as focal and generalized dystonia (Roubertie et al., 2008; Mantuano et al., 2010). In *tottering* mice stressors transform the mild baseline ataxia to overt abnormal movements that include severe dystonia of the trunk and limbs (Scholle et al., 2010). These symptoms are all cerebellar in origin since Purkinje cell specific knock-

down of P/Q-type calcium channels is sufficient to mimic the full spectrum of the *tottering*'s episodic symptoms (Mark et al., 2011).

To delineate the functional cerebellar correlates of ataxia, dyskinesia and dystonia, we examined the activity of Purkinje cells and deep cerebellar nuclei (DCN) neurons in awake *tottering* mice under baseline conditions and during caffeine-induced attacks. We found that as the severity of motor symptoms progressed from ataxia to dystonia, the firing of Purkinje cells and DCN neurons became correspondingly more bursty and erratic. The high frequency burst firing was necessary for the *tottering* mice to exhibit dystonia, and sufficient to mimic the severe symptoms of the attacks in wild type mice. We propose that a continuum in aberrant cerebellar output produces these diverse symptoms with ataxia associated with altered outputs that have diminished information content, whereas cerebellar induced dyskinesia and dystonia are caused by strong bursting activity of DCN neurons that act as erroneous command signals to instigate involuntary muscle contractions.

METHODS

Experiments were performed on 12 to 20 weeks old C57/BL6 and *tottering* mice inbred on the C57/BL6 strain for at least 25 generations. All experiments were in accord with the guidelines set by Albert Einstein College of Medicine.

Induction of attacks. To trigger an attack, 20 mg/kg caffeine (dissolved in 0.9% saline), a dose with 90% success rate for inducing attacks (Fureman et al., 2002), was injected in the backs of the mice subcutaneously with the aid of a previously implanted catheter. An attack was signaled by the presence of repetitive movements and sustained flexion of the limbs, abnormal extension of the tail and hunched posture of the trunk. Mice were administered a single injection per day independent of the outcome of the injection.

In vivo electrophysiology. Mice were prepared for chronic recordings under isofluorane anesthesia. A custom made "L"-shaped bracket was fixed onto the skull with three bone screws (Plastics One Inc.) and dental cement (M&S Dental Supply). A recording chamber 3 mm in diameter was drilled in the skull above the cerebellum, surrounded with dental cement and covered with surgi-foam (Ethicon) and bone wax (Ethicon). Following surgery, mice were allowed to recover for one week prior to the recording sessions.

To record neural activity, the head was immobilized by fixing the head bracket to the stereotaxic apparatus with a screw, the surgi-foam and bone wax were removed and the recording chamber filled with agar. Single-unit activity of DCN and Purkinje cells were recorded using a platinum-quartz electrode (2-3 MΩ, Thomas Recording GmbH), which was advanced into the cerebellum until either the Purkinje cell layer or DCN were

reached. Purkinje cells were identified by the brief pause in their activity following each complex spike, DCN cells by their location; lack of complex spikes and characteristic firing rate *in vivo* (Rowland and Jaeger, 2005; Hoebeek et al., 2008). Each cell was recorded for at least 10 minutes and attributed to the attack condition when motor symptoms could visually be clearly identified. If caffeine injection failed to induce an attack, cells recorded within a 50 minute time window following caffeine administration were attributed to the no attack condition. Extreme care was taken to closely monitor the motor behavior of mutant mice for spontaneous/stress-triggered attacks, which can occur under these experimental conditions. Cells were only classified as baseline when mice exhibited no motor symptoms for at least 10 minutes before and after recording.

Neural signals were band pass filtered (80 Hz – 20 kHz), amplified (2000×) and digitized (20 kHz) using a data acquisition card (PCI-MIO-16XE) and an in house written software based on LabView (National Instruments, Austin, TX). Waveforms were sorted using Offline Sorter software (Plexon Inc, Dallas, TX USA) using principle component analysis.

Chronic perfusion of the cerebellum. We chronically perfused the cerebellum as previously described (Calderon et al., 2011). Briefly, a single cannula (Plastics One) was stereotaxically implanted at midline (AP: -6.90 mm from Bregma and DV: 2 mm) and connected to an osmotic pump (model 1007D, 0.25 μl h^{-1}, Alzet). The pump was then placed under the skin on the back of the mice. Pumps were filled with solutions of cadmium chloride made in water and 0.01% methylene blue, which allowed for post-mortem examination of the perfusion site. Using this technique, we have shown that the slow rate of perfusion allows the drug to remain contained within the cerebellum, with its

concentration sharply dropping within 1 mm of the perfusion site (Calderon et al., 2011). Following surgery and every 12 hours thereafter, the long lasting NSAID pain reliever Flunixin was administered subcutaneously.

Acute injections of the cerebellum. For acute injections, a single injection cannula (Plastics One) was stereotaxically implanted at midline (AP: -6.90 mm from Bregma and DV: 2 mm) for NS309, and a double injection cannula at (ML: ± 0.75 mm, AP: -6.90 mm and DV: 2 mm) for $CdCl_2$. Following recovery, a total volume of 5 µl of the desired solution was injected over a period of 15 minutes using an automated pump (World Precision Instruments).

The behavior of each mouse before and after drug perfusion and injection was documented by video recordings.

Disability rating scale. The severity of motor dysfunction in the *tottering* and $CdCl_2$- and vehicle-perfused wild types was quantified according to a previously published scale (Weisz et al., 2005) as follows: 0 = normal motor behavior, 1 = slightly slowed or abnormal movements, 2 = mild impairments, limited ambulation unless disturbed, 3 = moderate impairment, limited ambulation even when disturbed, frequent abnormal postures, 4 = severe impairment, almost no ambulation, sustained abnormal postures, 5 = prolonged immobility in abnormal postures.

Analysis. The data analysis software was written using LabView. The mean firing rate was calculated as the number of spikes divided by the duration of the recording. The predominant firing rate was determined by calculating the reciprocal of the mode of the interspike interval distribution. The coefficient of variation of interspike intervals was calculated by dividing the standard deviations of interspike intervals by their means.

Statistical analyses were performed using Origin Software. Compiled data are reported as means ± SEM. Statistical significance was tested using one-way ANOVA followed by a 2-sample independent t-test and considered significant at $p < 0.05$.

Optogenetic Stimulation of DCN. 1.2 µl of virus (AAV2/1-Syn-ChR2-YFP; University of Pennsylvania Vector Core) was injected into the dentate nucleus of the cerebellum either unilaterally or bilaterally (AP: -6 mm; ML: ±2.3 mm; DV: -2.4 mm). An optical fiber (200 µm diameter, 0.48 NA, Thorlabs) was implanted to target the dentate nuclei. Light from a 473 nm or 450 nm laser (OEM Laser Systems) was delivered using fiberoptic ferrules (Kientec Systems) to the implanted fibers. Attenuation over the connector was measured before implantation to calculate output at the fiber tip during experiments. Stimuli were delivered as 10 ms light pulses at intensities ranging from <1 mW to 15 mW.

Piezoelectric Platform Recordings. A piezoelectric buzzer (Radioshack) was mounted on a platform. Signals were amplified 10x (Frequency Devices 902) and acquired at 20 kHz (National Instruments) in software written in Labview. Mice were stimulated every 5 seconds at intensities ranging from <1mW to 15mW. Individual trials were then averaged. Latency was calculated from the average trace.

RESULTS

Altered cerebellar output during attacks

In response to stress, caffeine or ethanol *tottering* mice exhibit highly stereotypic attacks that last 60 to 80 minutes (Fureman and Hess, 2005; Weisz et al., 2005; Shirley et al., 2008; Alvina and Khodakhah, 2010b). During an attack, motor dysfunction progresses from mild to severe ataxia, dyskinesia and ultimately to dystonia before abating (Shirley et al., 2008; Scholle et al., 2010).

From the finding that removing the cerebellum abolishes the expression of these episodes (Neychev et al., 2008), we inferred that attacks must be accompanied by an abnormal cerebellar output. To test this hypothesis, we examined the spontaneous activity of individual DCN neurons in awake head-restrained *tottering* mice under baseline conditions, and during attacks triggered by a single subcutaneous injection of 20 mg/kg caffeine (Figure 1a).

During the most severe phase of the attacks, 20 to 30 minutes after injection of caffeine, we found the regular baseline DCN activity to switch to a bursting firing pattern, characterized by frequent trains of high frequency spikes followed by pauses (Figure 1b). Quantitatively, this was evident as a marked increase in the mode of the interspike interval distribution, or predominant firing rate (mean predominant firing rate during baseline: 63 ± 4 spikes/s, n = 20; attack: 87 ± 6 spikes/s, n = 21; N = 5; $p < 0.001$; Figure 1d) and a significant increase in the coefficient of variation (CV) of interspike intervals (ISI) (standard deviation divided by the mean ISI), a common measure describing the variability in the timing of spikes within a spike train (mean CV of ISIs during baseline: 0.69 ± 0.05; attack: 1.26 ± 0.1; $p < 0.001$; Figure 1e). Interestingly, the

combination of high frequency bursts with relatively long intra-burst intervals yielded comparable average firing rates for baseline and attack conditions (average firing rate during baseline: 43 ± 4 spikes/s; attack: 38 ± 3 spikes/s; $p > 0.3$; Figure 1f).

At the concentration used, caffeine triggers attacks approximately 90% of the time, leaving the motor symptoms of the *tottering* mice unaltered when it fails to trigger one (Fureman et al., 2002). We used these failure trials (referred to as "no attack" throughout the text) as a control for any effect caffeine may have on DCN neurons independent from the expression of attacks. In the absence of an attack, caffeine did not alter the activity of DCN neurons (mean predominant firing rate: 67 ± 6 spikes/s, $p = 0.1$; coefficient of variation: 0.74 ± 0.09, $p > 0.1$; average firing rate: 49 ± 4 spikes/s, $p > 0.1$; $n = 4$, $N = 2$; all compared to baseline values; Figure 1 d - f).

In wild type mice, a single 20 mg/kg subcutaneous caffeine injection did not alter the activity of DCN neurons either (average firing rate before caffeine injection: 41 ± 4 spikes/s, $n = 27$; after caffeine: 42 ± 5 spikes/s, $n = 13$, $N = 7$; $p > 0.8$; mean predominant firing rate before caffeine injection: 59 ± 4 spikes/s; after caffeine: 56 ± 6 spikes/s; $p > 0.6$; ISI CV before caffeine injection: 0.72 ± 0.04; after caffeine: 0.68 ± 0.03; $p > 0.6$). These controls indicated that the changes seen in *tottering* mice were not caused by nonspecific pharmacologic actions of caffeine on DCN neurons but rather correlated with the presence of the attacks.

Three distinct nuclei provide the output from the cerebellum (Chan-Palay, 1977). Scrutiny of the recordings on the basis of the location of the neurons revealed that during caffeine-induced attacks the activity of *tottering* neurons in all three cerebellar nuclei was comparably affected (ISI CV in fastigial nucleus (baseline): 0.74 ± 0.06, $n = 12$; (attack):

1.28 ± 0.17, n = 11; $p < 0.001$; ISI CV in interposed nucleus (baseline): 0.66 ± 0.12, n = 6; (attack): 1.2 ± 0.17 n = 9, $p < 0.001$; ISI CV in lateral nucleus (baseline): 0.52, n = 2; (attack): 1.08; n = 2, N = 3; Figure 1g).

Electromyography (EMG) recordings from agonist and antagonist muscles in *tottering* mice unambiguously identify the motor symptoms at the peak of an attack as dystonia (Scholle et al., 2010). The data presented so far suggest that cerebellar-induced dystonia associates with highly erratic burst firing of cerebellar output neurons.

A feature of the attacks in the *tottering* is the progression of symptoms through stages of ataxia, dyskinesia and dystonia (Shirley et al., 2008; Scholle et al., 2010). The initial symptoms include a worsening of the baseline ataxia, followed by jerky movements of the limbs and finally at the peak of the attack paddling-like movements punctuated by sustained flexion of the limbs and abnormal posture of the trunk (Shirley et al., 2008; Scholle et al., 2010). Using a motor disability scoring scale, the severity of the symptoms can be reproducibly assessed (Weisz et al., 2005) (Figure 1i). We found both the ISI CV and the predominant firing rate of DCN neurons to tightly correlate with the severity of the symptoms, suggesting that dystonia, dyskinesia, and ataxia may all be manifestations of aberrant cerebellar output along a monotonic continuum (Figure 1j).

Burst firing in Purkinje cell activity during *tottering* dyskinesia attacks

Several lines of evidence suggest that Purkinje cells make a major contribution to the initiation of attacks in the *tottering*. First, as mentioned, targeted expression of the mutation in Purkinje cells recapitulates the spectrum of *tottering*'s syndrome (Mark et al., 2011). Second, removing Purkinje cells by crossing the *tottering* mouse with a mouse carrying the purkinje cell degeneration background mutation abolishes attacks (Campbell

et al., 1999). Moreover it has recently been shown that P/Q-type calcium channels do not play a major role in the regulation of pacemaking in DCN neurons (Alvina and Khodakhah, 2008), and *tottering* DCN neurons respond to Purkinje cell stimulation with the same efficacy as wild type ones (Hoebeek et al., 2008). These observations collectively suggest that the changes seen in the activity of DCN neurons might be driven by aberrant Purkinje cell input.

To test this hypothesis we recorded the activity of Purkinje cells in the awake head-restrained *tottering* under baseline and caffeine-triggered attack conditions (Figure 2 a, b). In agreement with a previous study (Chen et al., 2009) we found the average firing rate to remain the same during baseline and attacks (average firing rate during baseline: 52 ± 5 spikes/s, n = 13; attack: 54 ± 9 spikes/s, n = 14, N = 6, $p > 0.9$ relative to baseline; no attack: 63 ± 8 spikes/s, n = 5, N = 2, $p > 0.7$ relative to attack; Figure 2d). However, in the presence of caffeine-induced attacks the firing pattern of Purkinje cells transformed to high frequency burst firing. This is evident in the significantly increased predominant firing rate (baseline: 74 ± 4 spikes/s; attack: 180 ± 29 spikes/s; $p < 0.001$ relative to baseline; no attack: 85 ± 5 spikes/s; $p < 0.05$ relative to attack; Figure 2e) as well as the ISI CV (baseline: 0.66 ± 0.06; attack: 1.47 ± 0.14; $p < 0.001$ relative to baseline; no attack: 0.78 ± 0.23; $p < 0.05$ relative to attack; Figure 2f). The occurrence of high frequency burst firing was evident in the increased occurrence of interspike intervals of shortest and longest durations corresponding to bursts and pauses respectively (Figure 2g).

On the other hand, injection of caffeine did not alter the behavior of wild type Purkinje cells (average firing rate before caffeine: 44 ± 6 spikes/s, n =18; after caffeine:

48 ± 5 spikes/s, n = 12; N = 2; $p > 0.6$, mean predominant firing rate before caffeine: 77 ± 7 spikes/s; after caffeine: 75 ± 7 spikes/s; $p > 0.8$; ISI CV before caffeine: 0.78 ± 0.05; after caffeine: 0.74 ± 0.08; $p > 0.6$).

The change in the activity of Purkinje cells also correlated tightly with the severity of the symptoms (Figure 2 h, j). Additionally the changes in the activity of DCN neurons correlated well with those occurring at the level of Purkinje cells (Figure 2j), further corroborating that the abnormal cerebellar output patterns are driven by the burst firing of Purkinje cells.

Restoring the regularity of Purkinje cell activity alleviates dyskinesia attacks

If erratic Purkinje cell activity is necessary for the expression of dyskinesia in *tottering*, then one would expect restoring the regularity of Purkinje cell firing to abort the attacks and alleviate the episodic symptoms. To test this hypothesis we performed intracerebellar injections of a potent activator of small conductance calcium-activated potassium (SK) channels with the aid of a guide cannula surgically implanted on the skull overlying the cerebellum. We elected to use an SK channel activator based on the finding that in Purkinje cells, the calcium entry triggered by an action potential waveform is tightly coupled to the activation of SK channels (Womack and Khodakhah, 2002; Womack et al., 2004). By providing an outward current during the action potential afterhyperpolarization, the SK conductance regulates the duration of interspike intervals (Womack and Khodakhah, 2003). Thus, in these neurons, pharmacologically enhancing the activity of SK channels improves the precision of pacemaking (Walter et al., 2006; Alvina and Khodakhah, 2010b), and at high concentrations can even decrease their firing rate (Womack and Khodakhah, 2003).

To test whether intracerebellar injection of an SK channel activator would 1) abort an ongoing attack and 2) regularize the erratic *tottering* firing pattern *in vivo,* we first triggered an attack by systemically injecting mutants with caffeine. Once the start of an attack could unambiguously be detected, we injected 0.5 µg of NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime), an agent that potently and selectively increases the affinity of SK channels for calcium (Strobaek et al., 2004), into the cerebellum over a period of 15 minutes. Independent observers blinded to the conditions of the experiment scored the severity of dyskinesia from videos obtained 10 minutes after caffeine injection, as well as immediately after termination of NS309 injection.

We found intracerebellar administration of NS309 to significantly decrease the severity of motor symptoms from an average of 4.5 ± 0.2 to 2.1 ± 0.2 ($p < 0.001$; $N = 8$; Figure 3a). To determine if this was accompanied by changes in the behavior of Purkinje cells, we recorded their activity near the injection site (up to ±1 mm from the injection site). Intracerebellar injections of NS309 restored the activity of Purkinje cells by decreasing the predominant firing rate and CV of ISIs to their baseline values (mean predominant firing rate during an attack: 180 ± 29 spikes/s, $n = 14$, $N = 6$; after NS309: 71 ± 9 spikes/s, $n = 5$, $N = 3$; $p < 0.05$ relative to attack; CV of ISIs during an attack: 1.47 ± 0.14; after NS309: 0.74 ± 0.08; $p < 0.05$ relative to attack; Figure 3 b, c). On the other hand, NS309 injection did not associate with a change in the average firing rate (attack: 54 ± 9 spikes/s; after NS309: 45 ± 4 spikes/s; $p > 0.6$; Figure 3d).

Cadmium-induced erratic Purkinje cell activity is sufficient to induce dyskinesia

If the observed aberrant cerebellar activity underlies the expression of episodic motor symptoms, one would expect that inducing a similar pattern of Purkinje cell firing in wild type mice would result in the expression of similar motor deficits. Thus, to determine if aberrant cerebellar activity is sufficient to induce dyskinesia, we chronically perfused the cerebella of wild type mice with cadmium which is known to reliably induce burst firing *in vitro* (Walter et al., 2006).

We found the motor symptoms of wild types chronically perfused with 8 μg h^{-1} cadmium ($CdCl_2$) to progressively worsen over the course of 48 hours. Two days following the start of perfusion, they reached their maximum severity and remained stable until the pumps depleted 6 days following implantation. The initial symptoms included reduced coordination of movements and unsteady gait. Gradually the symptoms progressed to repetitive movement of the limbs, and frequent falling and finally sustained extensions and jerky movements of the limbs. We found that while mice receiving vehicle showed no signs of motor impairment, 48 hours after start of perfusion mice perfused with cadmium received disability scores that described the presence of severe motor dysfunction (vehicle: 0.1 ± 0.04, N = 9; $CdCl_2$: 3.8 ± 0.21, N = 9; $p < 0.001$; Figure 4a). Concurrently, the regular firing of Purkinje cells was transformed to burst firing similar to that seen in the *tottering* mice during attacks (predominant firing rate - vehicle: 68.2 ± 11 spikes/s, n = 5, N = 2; $CdCl_2$: 144 ± 11 spikes/s, n = 5, N = 3; $p < 0.05$; CV of ISIs - vehicle: 0.48 ± 0.04; $CdCl_2$: 0.89 ± 0.05; $p < 0.001$; mean firing rate- vehicle: 55 ± 7 spikes/s; $CdCl_2$: 66.2 ± 10 spikes/s; $p > 0.3$; Figure 4 b – e). These findings support the prediction that pharmacologically inducing erratic Purkinje cell firing in wild type mice

can induce motor symptoms comparable to those seen in the *tottering* mutant during dyskinesia attacks.

As a control for possible toxicity associated with long-term administration of $CdCl_2$, we injected 10 mM $CdCl_2$ acutely into the cerebella of a different set of wild types. The motor behavior of these mice was assessed similarly to the chronic perfusions 10 minutes prior to as well as 30 minutes after injecting $CdCl_2$. Acute injections of $CdCl_2$ were found to induce motor symptoms as reliably as chronic perfusions as indicated by the significant increase in the motor disability score (vehicle: 0.5 ± 0.1, $n = 4$, $N = 3$; $CdCl_2$: 4.7 ± 0.1, $n = 4$, $N = 3$; $p < 0.001$) indicating that the symptoms associated with chronic perfusions did not require the presence of toxic effects.

Optogenetic stimulation of DCN can induce both ataxia and involuntary muscle contractions

The data presented so far suggest a mechanism whereby ataxia, dyskinesia and dystonia would all be produced by an erratic cerebellar output driven by burst firing of Purkinje cells. Modest erratic Purkinje cell activity has been shown to reduce their ability to encode information leading to mild ataxia (Walter et al., 2006). It may be that a more moderate Purkinje cell burst firing might actually prevent their target DCN neurons from conveying any meaningful information at all. This latter scenario would be analogous to not having a cerebellum and thus would manifest as severe ataxia. Even more avid Purkinje cell burst firing is expected to, in turn, promote high frequency burst firing in a group of DCN neurons. This might then constitute erroneous command signals actually initiating involuntary muscle contractions yielding episodes of dyskinesia and dystonia. One approach to test these hypotheses would be to optogenetically promote aberrant

DCN activity and examine the consequences for motor function. An adeno-associated viral (AAV) vector containing channelrhodopsin-2 (ChR2) was stereotaxically injected into the DCN of wild type mice, and a fiber optic light guide implanted to target the transfected neurons. Activation of ChR2 with light generates an inward current that depolarizes the targeted DCN neurons, thus promoting their higher activity. The mice were placed on a platform coupled to a piezoelectric transducer. The piezoelectric transducer allowed for recording and subsequent quantification of the movements of the mice. We found that strong optogenetic stimulations (10 ms pulse of 473 nm light at 0.75 to 15 mW power) produced clear short latency (≈10 - 20 ms) movements (Figure 5 a, b). The latency of the stimulation-induced movement was a function of the stimulation intensity (Figure 5c). Stimulation of the DCN with a train of stimuli at 10 Hz resulted in repeated discrete movements, whereas at 20 Hz the animals assumed almost dystonic-like postures (Figure 5d). These results clearly demonstrate that DCN can initiate movement, and that its burst–like repeated activation could induce dystonic-like symptoms.

We next examined whether repeated activation of the DCN at intensities that did not generate movement resulted in ataxia by quantifying the duration of the time that the mice walked on a balance beam before falling. We found that stimulating the DCN at 20 Hz with a light intensity that did not produce any movement significantly reduced the length of time mice could stay on the balance beam (light off: 138.25 ± 47.2 s, n = 4 trials each in a total of N = 2 mice; light on: 8.04 ± 1.41 s, n = 17 trials total in N = 2 mice; $p < 0.001$). These data thus support the hypothesis that degrading the information content of DCN neurons by altering their firing rate can yield ataxia, and that strong DCN outputs can initiate muscle contractions, and if persistent, may lead to dystonia.

DISCUSSION

The most prevalent outcome of cerebellar dysfunction caused by its injury or neurodegeneration is ataxia. The symptoms associated with ataxia are in accord with the commonly ascribed function of the cerebellum, namely that under normal conditions it contributes to motor coordination but does not initiate it. A number of observations in patients and animals, however, have indicated that cerebellar dysfunction can also cause erroneous involuntary muscle contractions in the form of dyskinesia, and can even lead to prolonged co-contraction of agonist and antagonist muscle pairs as seen with dystonia. Here we took advantage of an animal model of EA2 to delineate the cerebellar output patterns that mediate these diverse motor symptoms. We found that ataxia, dyskinesia and dystonia represent a continuum of aberrant activity in the output of the cerebellum. As the severity of motor dysfunction progressed from poor movement coordination to sporadic muscle contractions and ultimately dystonia, the cerebellar output became correspondingly more erratic and discontinuous culminating in high frequency burst firing during dystonia. Our observations are in agreement with earlier studies showing that Purkinje cells are sufficient and required for initiation of attacks in EA2, and extend them by demonstrating that it is their continuous erratic firing that drive aberrant DCN activity to sustain the attacks. What remains to be established is the mechanism by which various triggers transform the activity of Purkinje cells into burst firing.

Tottering mice provide an invaluable opportunity for examining cerebellar dysfunction during a multitude of diverse motor symptoms

Although the primary manifestation of cerebellar dysfunction or atrophy is ataxia, numerous conditions and case reports associate it with a wide range of other symptoms.

For example dyskinesia, athetosis, and dystonia frequently manifest as prominent symptoms in conjunction with ataxia in ataxia-telangiectasia, ataxia-oculomotor apraxia types 1 and 2, dystonia with cerebellar atrophy (DYTCA), and in a large group of spinocerebellar ataxias including SCA1, 2, 3, 6, 7, 12, and 17 (Woods and Taylor, 1992; Modi et al., 2000; Schols et al., 2000; O'Hearn et al., 2001; Sethi and Jankovic, 2002; Hagenah et al., 2004; Wu et al., 2004; Le Ber et al., 2006; Zarubova and Ruzicka, 2006; Mariotti et al., 2007). Simply based on the assumption that dystonia is caused by basal ganglia dysfunction, the dystonia seen in these patients has routinely been attributed to basal ganglia dysfunction. However, while brain scans of these patients frequently show marked cerebellar atrophy that on occasion may be concomitant with mild cortical thinning, they rarely indicate any signs of basal ganglia atrophy or abnormality (O'Hearn et al., 2001; LeDoux and Brady, 2003; Mariotti et al., 2007). Moreover, in several patients focal dystonia has been reported to appear before ataxia, suggesting that dystonia may not have been a secondary outcome to the progression of degeneration (Munchau et al., 1999) and might have been caused by cerebellar dysfunction.

The cerebellum has also been implicated in generating diverse motor symptoms in episodic ataxia. Mutations in the *CACNA1A* gene encoding the P/Q-type calcium channels result in a number of allelic disorders including episodic ataxia type 2, SCA6, and familial hemiplegic migraine (Ophoff et al., 1996; Jodice et al., 1997). While the most common symptom of SCA6 and EA2 is ataxia, in both disorders dystonia can also be present (Guida et al., 2001; Sethi and Jankovic, 2002; Muzaimi et al., 2003). For example, some EA2 patients suffer from interictal focal dystonia, and in some others the episodic attacks are accompanied by cervical dystonia (paroxysmal torticollis) (Roubertie

et al., 2008; Cuenca-Leon et al., 2009; Mantuano et al., 2010). The presence of faithful animal models has facilitated examination of the origins of motor dysfunction in EA2. The *tottering* mice suffer from a spontaneous point mutation that, similar to comparable mutations in EA2 patients, reduces the P/Q-type calcium current density in Purkinje cells (Wakamori et al., 1998; Rajakulendran et al., 2010b). These mice show motor symptoms that closely mimic the disorder in EA2 patients (Fureman et al., 2002; Pietrobon, 2002), and they also respond to the same drugs that lessen the severity of motor dysfunction in EA2 patients (Strupp et al., 2004; Weisz et al., 2005; Strupp et al., 2011). Numerous studies have provided strong evidence in support of the hypothesis that the motor dysfunction seen during the attacks in the *tottering* is cerebellar in origin (Neychev et al., 2008) and specifically require cerebellar Purkinje cells (Campbell et al., 1999; Mark et al., 2011). The attacks in the *tottering* are highly reproducible and inevitably progress to trunk and limb dystonia before abating (Scholle et al., 2010). This provided an invaluable opportunity for scrutinizing the cerebellar output patterns associated with each of these symptoms when attacks were triggered by caffeine, a common trigger in EA2 patients and also in the *tottering* mice (Ptacek, 1997; Fureman et al., 2002).

Ataxia caused by alterations in cerebellar circuitry and physiology

Prior studies in several mouse models of EA2 have demonstrated that the reduction in the P/Q-type calcium current reduces the precision of Purkinje cell pacemaking (Hoebeek et al., 2005; Walter et al., 2006; Alvina and Khodakhah, 2010a). The loss in the precision of Purkinje cell pacemaking in turn reduces the quality of the synaptically-encoded information in the activity of Purkinje cells (Hoebeek et al., 2005; Walter et al., 2006) and contributes to the baseline ataxia seen in a number of mouse

models of EA2, including the *tottering* mice (Walter et al., 2006). Such degradation in the information encoded by Purkinje cells is also seen in EA1 where inhibitory synaptic inputs to Purkinje cells are noisy and erratic (Maylie et al., 2002) and also in SCA3 where many Purkinje cells are silent due to depolarization block and those that fire are more erratic (Shakkottai et al., 2011). However, prior to this study little was understood regarding the changes that occur in the activity of Purkinje cells during episodic attacks of motor dysfunction in EA2. We found that during attacks as the severity of ataxia worsened, the firing of Purkinje cells in awake *tottering* mice became more erratic. This finding is in agreement with the prediction that further reductions in the ability of Purkinje cells to encode motor-related information yields more severe ataxic symptoms, perhaps culminating in the severe ataxia which is associated with acute removal of the cerebellum.

Cerebellar-induced muscle contractions in dyskinesia and dystonia

While ataxia is associated with loss of information in the activity of DCN neurons, we propose that cerebellar-induced symptoms that manifest as involuntary muscle contractions represent conditions during which the cerebellum sends out erroneous command signals. In this case the high frequency burst firing of DCN neurons might erroneously force downstream motor areas to action. It is intriguing to note that high frequency DCN burst firing was observed in recordings from three patients with congenital athetosis (Slaughter et al., 1970), and that several case reports have documented the efficacy of lesioning the cerebellar nuclei as a treatment for dyskinetic movements or dystonia in humans (Heimburger, 1967; Zervas et al., 1967; Fraioli and Guidetti, 1975).

An issue that requires discussion is the seemingly dissonant finding that cerebellar dysfunction can initiate muscle contractions to generate dyskinesia and dystonia. As discussed earlier, it is widely accepted that while the cerebellum contributes to motor coordination it does not initiate movement. Yet it must be emphasized that the assertion that the cerebellum does not initiate muscle contractions under normal conditions does not mean that it is unable to do so. Indeed, numerous studies have shown that stimulation of cerebellar output nuclei can produce an assortment of movements in diverse animals ranging from birds and alligators to cats and rats, including nonhuman primates (Clark, 1939; Goodman and Simpson, 1960; Schultz et al., 1979). There are even a number of studies reporting that stimulation of the cerebellum in humans can initiate movement and produce sustained muscle contractions (Nashold and Slaughter, 1969). A criticism often levied against these studies has been that electrical stimulation of cerebellar nuclei would also activate the spinocerebellar and corticocerebellar mossy fibers. It is therefore plausible that the muscle contractions seen with electrical stimulations were a consequence of activation of mossy fibers rather than cerebellar output neurons. However, many of the investigators were fully cognizant of this caveat and had optimized the experimental conditions to minimize these unwanted mossy fiber stimulations. Moreover, here we report that optogenetic stimulation of cerebellar nuclei can also initiate movement and cause muscle contractions. Because in our experiments the light sensitive channels were only expressed in cerebellar nuclear neurons and were not present in the mossy fibers, our data unequivocally demonstrate that the cerebellum can initiate movement and corroborate the earlier observations made with electrical stimulations.

It is worth noting that the data presented are not at odds with the assertion that under normal conditions the cerebellum does not initiate movement. Instead, they simply demonstrate that direct strong activation of cerebellar output nuclei has the ability to do so. While it might perhaps be unlikely that such output patterns occur during normal function, pathological conditions may result in high frequency burst firing of cerebellar output neurons that resemble the pattern achieved by their direct electrical or optogenetic stimulation. We propose that such transient changes in the cerebellar output send erroneous signals to muscles to cause involuntary muscle contractions manifesting as dyskinesia and dystonia. Such cerebellar-initiated muscle contractions can be implemented by at least three cerebellar pathways: 1) cerebello-thalamo-cortical pathway (Rispal-Padel et al., 1981), 2) a disynaptic cerebello-basal ganglia pathway (Hoshi et al., 2005), and 3) by direct connections between the cerebellum and the descending pathways (Asanuma et al., 1980).

Cerebellar dysfunction in migraine, seizures, and cognitive and psychiatric disorders

As alluded to earlier, cerebellar dysfunction has also been implicated in a diverse group of neurologic and psychiatric disorders. Familial hemiplegic migraine, for example, is allelic to EA2 and SCA6 (Ophoff et al., 1996; Jodice et al., 1997), and is often triggered by the same stressors that trigger attacks in EA2 (Ptacek, 1997). In addition to migraine and seizures, the FHM phenotype includes transient or permanent cerebellar signs such as nystagmus, ataxia and dysarthria (Russell and Ducros, 2011). Indeed around 20% of hemiplegic patients show permanent mild cerebellar deficits (Russell and Ducros, 2011), and many EA2 patients frequently suffer from migraine (Jen

et al., 2004). In addition to the cerebellum being directly involved in cognition, cerebellar dysfunction may give rise to cognitive and psychiatric symptoms, or episodic seizure and migraine, by providing disrupting aberrant inputs to the basal ganglia and cortical structures. Ultimately, examination of phenotypically faithful animal models of these disorders may provide the needed platforms for testing these hypotheses.

REFERENCES

Alvina K, Khodakhah K (2008) Selective regulation of spontaneous activity of neurons of the deep cerebellar nuclei by N-type calcium channels in juvenile rats. The Journal of physiology 586:2523-2538.

Alvina K, Khodakhah K (2010a) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Alvina K, Khodakhah K (2010b) The therapeutic mode of action of 4-aminopyridine in cerebellar ataxia. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7258-7268.

Asanuma C, Thach WT, Jones EG (1980) Nucleus interpositus projection to spinal interneurons in monkeys. Brain research 191:245-248.

Calderon DP, Fremont R, Kraenzlin F, Khodakhah K (2011) The neural substrates of rapid-onset Dystonia-Parkinsonism. Nature neuroscience 14:357-365.

Campbell DB, North JB, Hess EJ (1999) Tottering mouse motor dysfunction is abolished on the Purkinje cell degeneration (pcd) mutant background. Experimental neurology 160:268-278.

Chan-Palay V (1977) In: Cerebellar dentate nucleus. Organization, cytology, and transmitters. Berlin: Springer

Chen G, Popa LS, Wang X, Gao W, Barnes J, Hendrix CM, Hess EJ, Ebner TJ (2009) Low-frequency oscillations in the cerebellar cortex of the tottering mouse. Journal of neurophysiology 101:234-245.

Clark SL (1939) Responses following electrical stimulation of the cerebellar cortex in the normal cat. The Journal of physiology 2:19-35.

Cuenca-Leon E, Banchs I, Serra SA, Latorre P, Fernandez-Castillo N, Corominas R, Valverde MA, Volpini V, Fernandez-Fernandez JM, Macaya A, Cormand B (2009) Late-onset episodic ataxia type 2 associated with a novel loss-of-function mutation in the CACNA1A gene. Journal of the neurological sciences 280:10-14.

Devanagondi R, Egami K, LeDoux MS, Hess EJ, Jinnah HA (2007) Neuroanatomical substrates for paroxysmal dyskinesia in lethargic mice. Neurobiology of disease 27:249-257.

Fatemi SH et al. (2012) Consensus Paper: Pathological Role of the Cerebellum in Autism. Cerebellum.

Fletcher NA, Stell R, Harding AE, Marsden CD (1988) Degenerative cerebellar ataxia and focal dystonia. Movement disorders : official journal of the Movement Disorder Society 3:336-342.

Flourens P (1824) Recherches expérimentales sur les propriétés et les fonctions du système nerveux dans les animaux vertébrés. Paris: Crevot

Fraioli B, Guidetti (1975) Effects of stereotactic lesions of the dentate nucleus of the cerebellum in man. Applied neurophysiology 38:81-90.

Fureman BE, Hess EJ (2005) Noradrenergic blockade prevents attacks in a model of episodic dysfunction caused by a channelopathy. Neurobiology of disease 20:227-232.

Fureman BE, Jinnah HA, Hess EJ (2002) Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. Pharmacology, biochemistry, and behavior 73:631-637.

Goodman DC, Simpson JT, Jr. (1960) Cerebellar stimulation in the unrestrained and unanesthetized alligator. The Journal of comparative neurology 114:127-135.

Guida S, Trettel F, Pagnutti S, Mantuano E, Tottene A, Veneziano L, Fellin T, Spadaro M, Stauderman K, Williams M, Volsen S, Ophoff R, Frants R, Jodice C, Frontali M, Pietrobon D (2001) Complete loss of P/Q calcium channel activity caused by a CACNA1A missense mutation carried by patients with episodic ataxia type 2. American journal of human genetics 68:759-764.

Hagenah JM, Zuhlke C, Hellenbroich Y, Heide W, Klein C (2004) Focal dystonia as a presenting sign of spinocerebellar ataxia 17. Movement disorders : official journal of the Movement Disorder Society 19:217-220.

Heimburger RF (1967) Dentatectomy in the treatment of dyskinetic disorders. Confinia neurologica 29:101-106.

Ho BC, Mola C, Andreasen NC (2004) Cerebellar dysfunction in neuroleptic naive schizophrenia patients: clinical, cognitive, and neuroanatomic correlates of cerebellar neurologic signs. Biological psychiatry 55:1146-1153.

Hoebeek FE, Khosrovani S, Witter L, De Zeeuw CI (2008) Purkinje cell input to cerebellar nuclei in tottering: ultrastructure and physiology. Cerebellum 7:547-558.

Hoebeek FE, Stahl JS, van Alphen AM, Schonewille M, Luo C, Rutteman M, van den Maagdenberg AM, Molenaar PC, Goossens HH, Frens MA, De Zeeuw CI (2005) Increased noise level of purkinje cell activities minimizes impact of their modulation during sensorimotor control. Neuron 45:953-965.

Hoshi E, Tremblay L, Feger J, Carras PL, Strick PL (2005) The cerebellum communicates with the basal ganglia. NatNeurosci 8:1491-1493.

Ito M (1984) The Cerebellum and Neural Control. New York: Raven.

Jen J, Kim GW, Baloh RW (2004) Clinical spectrum of episodic ataxia type 2. Neurology 62:17-22.

Jodice C, Mantuano E, Veneziano L, Trettel F, Sabbadini G, Calandriello L, Francia A, Spadaro M, Pierelli F, Salvi F, Ophoff RA, Frants RR, Frontali M (1997) Episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6) due to CAG repeat expansion in the CACNA1A gene on chromosome 19p. Human molecular genetics 6:1973-1978.

Le Ber I, Clot F, Vercueil L, Camuzat A, Viemont M, Benamar N, De Liege P, Ouvrard-Hernandez AM, Pollak P, Stevanin G, Brice A, Durr A (2006) Predominant dystonia with marked cerebellar atrophy: a rare phenotype in familial dystonia. Neurology 67:1769-1773.

LeDoux MS, Brady KA (2003) Secondary cervical dystonia associated with structural lesions of the central nervous system. Movement disorders : official journal of the Movement Disorder Society 18:60-69.

LeDoux MS, Lorden JF, Ervin JM (1993) Cerebellectomy eliminates the motor syndrome of the genetically dystonic rat. Experimental neurology 120:302-310.

Lees A (1990) Dystonia and cerebellar ataxia. Movement disorders : official journal of the Movement Disorder Society 5:178.

Mantuano E, Romano S, Veneziano L, Gellera C, Castellotti B, Caimi S, Testa D, Estienne M, Zorzi G, Bugiani M, Rajabally YA, Barcina MJ, Servidei S, Panico A, Frontali M, Mariotti C (2010) Identification of novel and recurrent CACNA1A gene mutations in fifteen patients with episodic ataxia type 2. Journal of the neurological sciences 291:30-36.

Mariotti C, Alpini D, Fancellu R, Soliveri P, Grisoli M, Ravaglia S, Lovati C, Fetoni V, Giaccone G, Castucci A, Taroni F, Gellera C, Di Donato S (2007) Spinocerebellar ataxia type 17 (SCA17): oculomotor phenotype and clinical characterization of 15 Italian patients. Journal of neurology 254:1538-1546.

Mark MD, Maejima T, Kuckelsberg D, Yoo JW, Hyde RA, Shah V, Gutierrez D, Moreno RL, Kruse W, Noebels JL, Herlitze S (2011) Delayed postnatal loss of P/Q-type calcium channels recapitulates the absence epilepsy, dyskinesia, and ataxia phenotypes of genomic Cacna1a mutations. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4311-4326.

Maylie B, Bissonnette E, Virk M, Adelman JP, Maylie JG (2002) Episodic ataxia type 1 mutations in the human Kv1.1 potassium channel alter hKvbeta 1-induced N-type inactivation. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4786-4793.

Modi G, Modi M, Martinus I, Rodda J, Saffer D (2000) The clinical and genetic characteristics of spinocerebellar ataxia type 7 (SCA 7) in three Black South African families. Acta neurologica Scandinavica 101:177-182.

Munchau A, Dressler D, Bhatia KP, Vogel P, Zuhlke C (1999) Machado-Joseph disease presenting as severe generalised dystonia in a German patient. Journal of neurology 246:840-842.

Muzaimi MB, Wiles CM, Robertson NP, Ravine D, Compston DA (2003) Task specific focal dystonia: a presentation of spinocerebellar ataxia type 6. Journal of neurology, neurosurgery, and psychiatry 74:1444-1445.

Nashold BS, Jr., Slaughter DG (1969) Effects of stimulating or destroying the deep cerebellar regions in man. Journal of neurosurgery 31:172-186.

Neychev VK, Fan X, Mitev VI, Hess EJ, Jinnah HA (2008) The basal ganglia and cerebellum interact in the expression of dystonic movement. Brain : a journal of neurology 131:2499-2509.

Nicolson RI, Fawcett AJ, Dean P (2001) Developmental dyslexia: the cerebellar deficit hypothesis. Trends in neurosciences 24:508-511.

O'Hearn E, Holmes SE, Calvert PC, Ross CA, Margolis RL (2001) SCA-12: Tremor with cerebellar and cortical atrophy is associated with a CAG repeat expansion. Neurology 56:299-303.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. Cell 87:543-552.

Pietrobon D (2002) Calcium channels and channelopathies of the central nervous system. Molecular neurobiology 25:31-50.

Ptacek LJ (1997) Channelopathies: ion channel disorders of muscle as a paradigm for paroxysmal disorders of the nervous system. Neuromuscular disorders : NMD 7:250-255.

Rajakulendran S, Schorge S, Kullmann DM, Hanna MG (2010a) Dysfunction of the Ca(V)2.1 calcium channel in cerebellar ataxias. F1000 biology reports 2.

Rajakulendran S, Graves TD, Labrum RW, Kotzadimitriou D, Eunson L, Davis MB, Davies R, Wood NW, Kullmann DM, Hanna MG, Schorge S (2010b) Genetic and functional characterisation of the P/Q calcium channel in episodic ataxia with epilepsy. JPhysiol 588:1905-1913.

Rispal-Padel L, Cicirata F, Pons C (1981) Contribution of the dentato-thalamo-cortical system to control of motor synergy. NeurosciLett 22:137-144.

Roubertie A, Echenne B, Leydet J, Soete S, Krams B, Rivier F, Riant F, Tournier-Lasserve E (2008) Benign paroxysmal tonic upgaze, benign paroxysmal torticollis, episodic ataxia and CACNA1A mutation in a family. Journal of neurology 255:1600-1602.

Rowland NC, Jaeger D (2005) Coding of tactile response properties in the rat deep cerebellar nuclei. Journal of neurophysiology 94:1236-1251.

Russell MB, Ducros A (2011) Sporadic and familial hemiplegic migraine: pathophysiological mechanisms, clinical characteristics, diagnosis, and management. Lancet neurology 10:457-470.

Sadnicka A, Hoffland BS, Bhatia KP, van de Warrenburg BP, Edwards MJ (2012) The cerebellum in dystonia - help or hindrance? Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology 123:65-70.

Scholle HC, Jinnah HA, Arnold D, Biedermann FH, Faenger B, Grassme R, Hess EJ, Schumann NP (2010) Kinematic and electromyographic tools for characterizing movement disorders in mice. Movement disorders : official journal of the Movement Disorder Society 25:265-274.

Schols L, Peters S, Szymanski S, Kruger R, Lange S, Hardt C, Riess O, Przuntek H (2000) Extrapyramidal motor signs in degenerative ataxias. Archives of neurology 57:1495-1500.

Schultz W, Montgomery EB, Jr., Marini R (1979) Proximal limb movements in response to microstimulation of primate dentate and interpositus nuclei mediated by brain-stem structures. Brain : a journal of neurology 102:127-146.

Sethi KD, Jankovic J (2002) Dystonia in spinocerebellar ataxia type 6. Movement disorders : official journal of the Movement Disorder Society 17:150-153.

Shakkottai VG, do Carmo Costa M, Dell'Orco JM, Sankaranarayanan A, Wulff H, Paulson HL (2011) Early changes in cerebellar physiology accompany motor dysfunction in the polyglutamine disease spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:13002-13014.

Shirley TL, Rao LM, Hess EJ, Jinnah HA (2008) Paroxysmal dyskinesias in mice. Movement disorders : official journal of the Movement Disorder Society 23:259-264.

Slaughter DG, Nashold BS, Jr., Somjen GG (1970) Electrical recording with micro- and macroelectrodes from the cerebellum of man. Journal of neurosurgery 33:524-528.

Strobaek D, Teuber L, Jorgensen TD, Ahring PK, Kjaer K, Hansen RS, Olesen SP, Christophersen P, Skaaning-Jensen B (2004) Activation of human IK and SK Ca2+ - activated K+ channels by NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime). Biochimica et biophysica acta 1665:1-5.

Strupp M, Kalla R, Dichgans M, Freilinger T, Glasauer S, Brandt T (2004) Treatment of episodic ataxia type 2 with the potassium channel blocker 4-aminopyridine. Neurology 62:1623-1625.

Strupp M, Kalla R, Claassen J, Adrion C, Mansmann U, Klopstock T, Freilinger T, Neugebauer H, Spiegel R, Dichgans M, Lehmann-Horn F, Jurkat-Rott K, Brandt T, Jen JC, Jahn K (2011) A randomized trial of 4-aminopyridine in EA2 and related familial episodic ataxias. Neurology 77:269-275.

Wakamori M, Yamazaki K, Matsunodaira H, Teramoto T, Tanaka I, Niidome T, Sawada K, Nishizawa Y, Sekiguchi N, Mori E, Mori Y, Imoto K (1998) Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. The Journal of biological chemistry 273:34857-34867.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. Nature neuroscience 9:389-397.

Weisz CJ, Raike RS, Soria-Jasso LE, Hess EJ (2005) Potassium channel blockers inhibit the triggers of attacks in the calcium channel mouse mutant tottering. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4141-4145.

Womack MD, Khodakhah K (2002) Characterization of large conductance Ca2+-activated K+ channels in cerebellar Purkinje neurons. The European journal of neuroscience 16:1214-1222.

Womack MD, Khodakhah K (2003) Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar Purkinje neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:2600-2607.

Womack MD, Chevez C, Khodakhah K (2004) Calcium-activated potassium channels are selectively coupled to P/Q-type calcium channels in cerebellar Purkinje neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:8818-8822.

Woods CG, Taylor AM (1992) Ataxia telangiectasia in the British Isles: the clinical and laboratory features of 70 affected individuals. The Quarterly journal of medicine 82:169-179.

Wu YR, Lee-Chen GJ, Lang AE, Chen CM, Lin HY, Chen ST (2004) Dystonia as a presenting sign of spinocerebellar ataxia type 1. Movement disorders : official journal of the Movement Disorder Society 19:586-587.

Zarubova K, Ruzicka E (2006) Cervical dystonia in spinocerebellar ataxia type 2. Movement disorders : official journal of the Movement Disorder Society 21:1295-1296.

Zervas NT, Horner FA, Pickren KS (1967) The treatment of dyskinesia by stereotxic dentatectomy. Confinia neurologica 29:93-100.

FIGURES

FIGURE 1

The erratic activity of *tottering* DCN neurons correlates with the severity of motor symptoms.

(a) Single-unit activity of *tottering* DCN neurons was monitored during baseline and in the presence (attack) and absence (no attack) of caffeine-induced dyskinesia episodes.

(b) Raw data illustrating the characteristic behavior of a *tottering* DCN neuron during baseline and in the presence of caffeine-induced attacks.

(c) Interspike interval distribution histogram of a *tottering* DCN neuron under baseline conditions and during an attack.

(d-f) Individual values and mean ± S.E.M of the interspike interval coefficient of variation, predominant firing rate, and mean firing rate of *tottering* DCN neurons under baseline conditions (baseline), during caffeine induced attacks (attack) and on occasions when caffeine failed to produce an attack (no attack).

(g) Individual values and mean ± S.E.M of ISI CV of DCN cells in individual deep cerebellar nuclei.

(h) Cumulative occurrence of interspike intervals of *tottering* DCN neurons. During attacks, a larger fraction of interspike intervals occur at short durations.

(i) Average disability score with respect to the time of caffeine injection.

(j) Average ISI CV and predominant firing rate of DCN neurons as a function of the average disability score.

* denotes $p < 0.05$, ** denotes $p < 0.001$, n.s. denotes “not significant”.

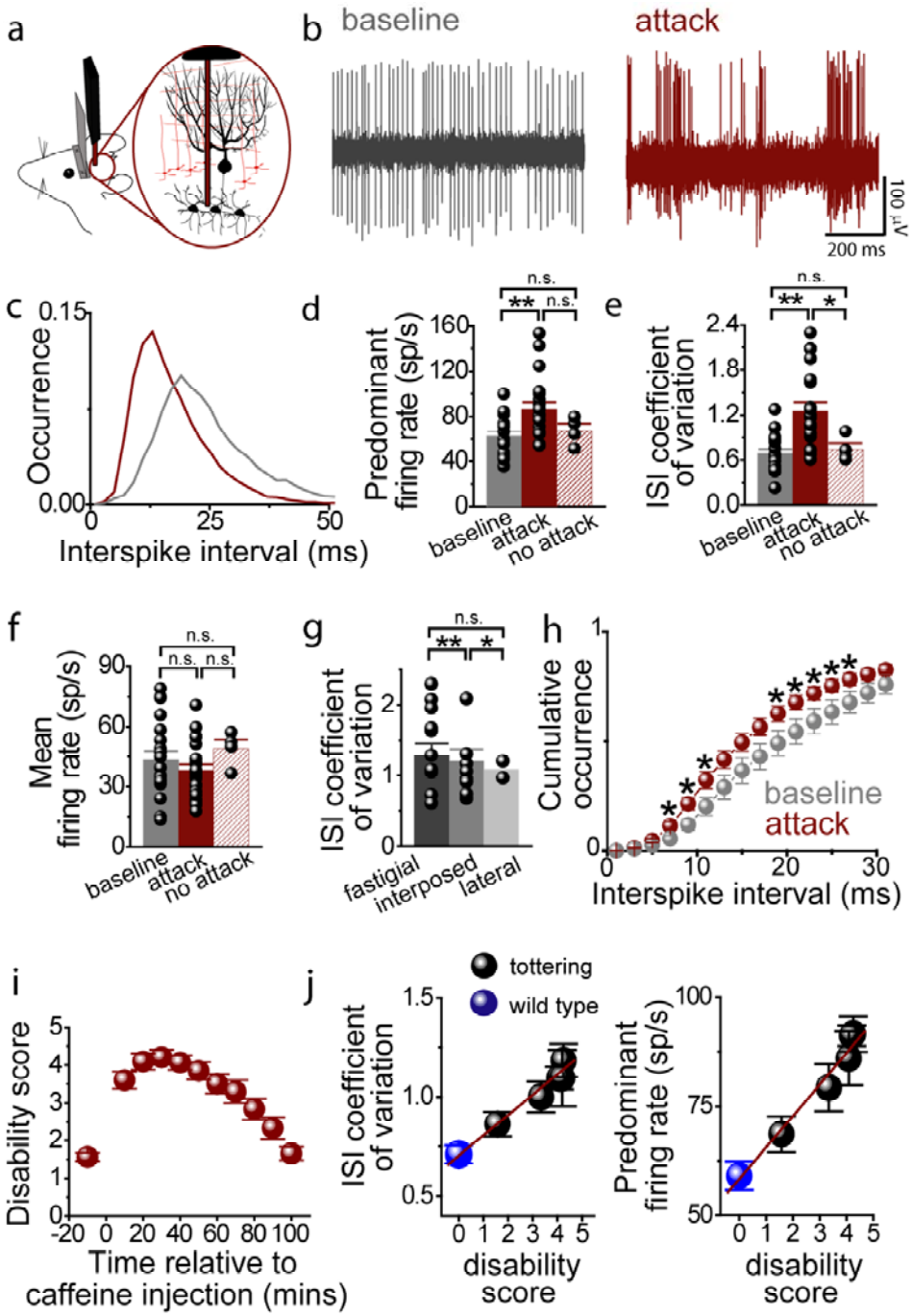

a
b
baseline
attack
100 μV
200 ms
c
Occurrence
0.15
0.00
0
25
50
Interspike interval (ms)
d
Predominant firing rate (sp/s)
n.s.
**
n.s.
baseline
attack
no attack
e
ISI coefficient of variation
n.s.
**
*
baseline
attack
no attack
f
Mean firing rate (sp/s)
n.s.
n.s.
n.s.
baseline
attack
no attack
g
ISI coefficient of variation
n.s.
**
*
fastigial
interposed
lateral
h
Cumulative occurrence
baseline
attack
Interspike interval (ms)
i
Disability score
Time relative to caffeine injection (mins)
j
tottering
wild type
ISI coefficient of variation
disability score
Predominant firing rate (sp/s)
disability score

FIGURE 2

The erratic activity of *tottering* Purkinje cells correlates with the severity of motor symptoms.

(a) Single-unit activity of Purkinje cells was monitored *in vivo* in the awake *tottering* mouse during baseline and in the presence and absence of caffeine-induced attacks.

(b) Activity of a typical Purkinje cell in the awake *tottering* mouse during baseline and attack conditions. Note the increased irregularity of the firing pattern during attacks. * in the raw trace denotes a complex spike.

(c) Interspike interval distribution histogram of a single Purkinje cell during baseline and attack.

(d - f) Inidividual values and mean ± S.E.M of the firing rate, interspike interval coefficient of variation and predominant firing rate of Purkinje cells under baseline conditions (baseline), during caffeine induced attacks (attack) and on occasions when caffeine failed to produce and attack (no attack).

(h) Average ISI CV and predominant firing rate of Purkinje cells as a function of the average disability score.

(i) Average ISI CV of DCN neurons with respect to the average ISI of Purkinje cells corresponding to the same disability score.

* denotes $p < 0.05$, ** denotes $p < 0.001$, n.s. denotes “not significant”.

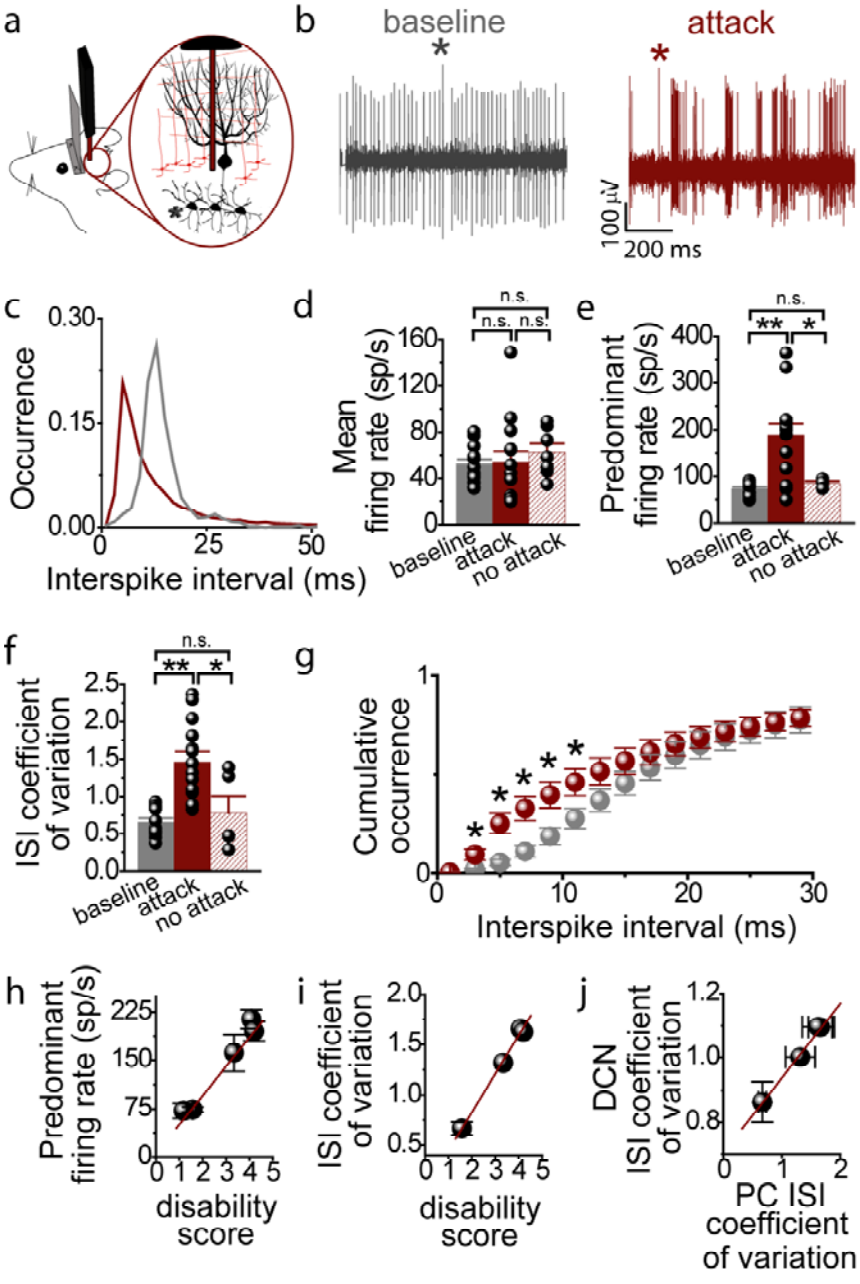

a
b
baseline
attack
100 μV
200 ms
c
Occurrence
Interspike interval (ms)
d
Mean firing rate (sp/s)
n.s.
baseline
attack
no attack
e
Predominant firing rate (sp/s)
**
*
f
ISI coefficient of variation
g
Cumulative occurrence
Interspike interval (ms)
h
Predominant firing rate (sp/s)
disability score
i
ISI coefficient of variation
disability score
j
DCN ISI coefficient of variation
PC ISI coefficient of variation

FIGURE 3

Restoring Purkinje cell regularity alleviates dyskinesia in the *tottering* mice.

(a) The severity of the motor symptoms of *tottering* mice during caffeine-induced attacks, before and after acute cerebellar injection of 0.5 μg h^{-1} of NS309.

(b - d) Individual and average values for the predominant firing rate and CV of ISIs and the mean firing rate of *tottering* Purkinje cells during baseline and attacks, and after acute injection of 0.5 μg h^{-1} NS309.

* denotes $p < 0.05$, ** denotes $p < 0.001$.

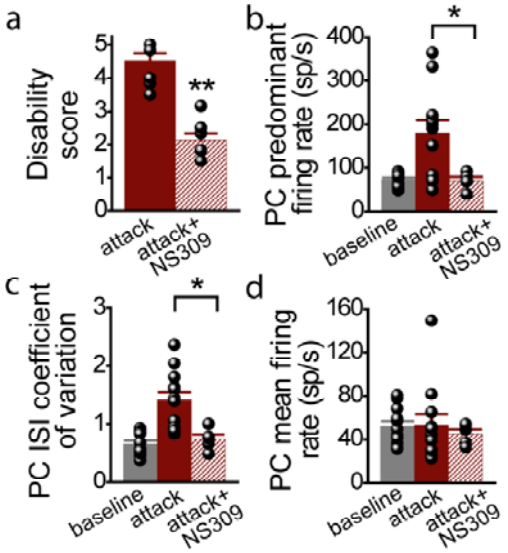

a
Disability score
5
4
3
2
1
0
**
attack
attack+ NS309
b
PC predominant firing rate (sp/s)
400
300
200
100
0
*
baseline
attack
attack+ NS309
c
PC ISI coefficient of variation
3
2
1
0
*
baseline
attack
attack+ NS309
d
PC mean firing rate (sp/s)
160
120
80
40
0
baseline
attack
attack+ NS309

FIGURE 4

Chronic *in vivo* blockade of cerebellar calcium channels results in dyskinesia in wild type mice.

(a) Disability scores of wild type mice 48 hours after start of chronic perfusion of 8 μg h^{-1} $CdCl_2$ or vehicle into the vermal region of the cerebellum.

(b) Activity of a typical Purkinje cell in the $CdCl_2$ and vehicle perfused mice.
* in the raw trace denotes a complex spike.

(c - e) Individual values and mean ± S.E.M of the predominant firing rate, CV of ISIs and the mean firing rate of Purkinje cells for vehicle and $CdCl_2$ perfused mice.

* denotes $p<0.05$; ** denotes $p < 0.001$.

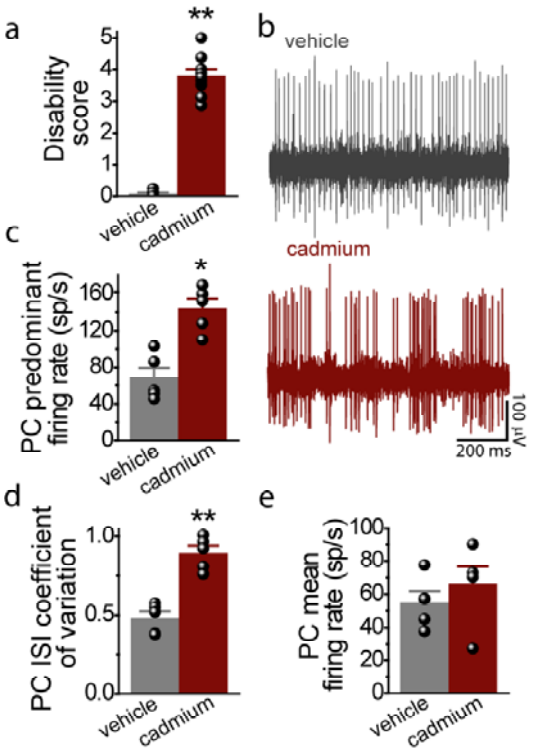

a
Disability score
**
vehicle
cadmium
b
vehicle
cadmium
100 µV
200 ms
c
PC predominant firing rate (sp/s)
*
vehicle
cadmium
d
PC ISI coefficient of variation
**
vehicle
cadmium
e
PC mean firing rate (sp/s)
vehicle
cadmium

FIGURE 5

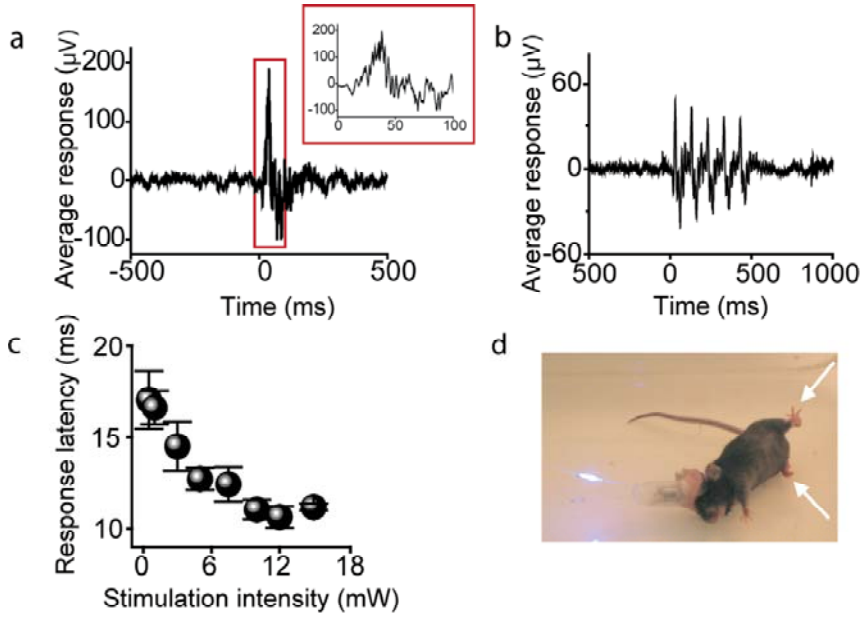
Optogenetic unilateral activation of the cerebellar dentate nucleus elicits movements.

(a) The piezoelectric signal reporting vibrations of platform on which the mice were standing in response to optogenetic activation (10 ms pulse at 10 mW) of the dentate nucleus. The trace is an average of ≈100 trials. Inset shows the same response in an expanded timescale.

(b) Repetitive optogenetic activation (five 10 ms pulses, 6 mW intensity, 10 Hz) of the dentate nucleus produces movements that robustly follow the stimuli (the trace is an average of ≈100 trials).

(c) Latency of initiation of movement by optogenetic stimulation of the dentate nucleus as a function of the stimulus intensity.

(d) Repetitive stimulation of the dentate nucleus (20 Hz, 6 mW) produces sustained contraction of muscles resembling dystonia.


a
Average response (μV)
200
100
0
-100
-500
0
500
Time (ms)
200
100
0
-100
0
50
100
b
Average response (μV)
60
0
-60
500
0
500
1000
Time (ms)
c
Response latency (ms)
20
15
10
0
6
12
18
Stimulation intensity (mW)
d

CHAPTER III:

NOREPINEPHRINE-INDUCED MODULATION OF SK CHANNELS AS A PUTATIVE MECHANISM FOR THE INDUCTION OF DYSKINESIA ATTACKS IN A MOUSE MODEL OF EPISODIC ATAXIA TYPE 2

Esra Tara, Christopher Chen[1] and Kamran Khodakhah

[1] Christopher Chen contributed to Figure 5

ABSTRACT

Episodic channelopathies are characterized by the expression of symptoms during discrete attacks superimposed on an unremarkable baseline phenotype. A common feature of these disorders is that in all of them attacks are induced by the same set of physical or psychological stressors, suggesting the existence of a shared mechanism for attack initiation. Episodic ataxia type 2 (EA2) is one such disorder that arises from mutations in the P/Q-type voltage-gated calcium channels. In this disorder a mild baseline ataxia is interrupted by attacks of severe motor dysfunction. We find that in the *tottering* mouse model of EA2 caffeine-induced attacks of dyskinesia are characterized by high frequency burst firing of cerebellar Purkinje. The mechanism by which triggers alter the activity of Purkinje cells is unknown; however restoring the baseline regularity of Purkinje cell firing using SK channel activators can abolish attacks. Here we show that Purkinje cell burst firing and an altered cerebellar output similarly accompany stress-triggered attacks from the cerebellum. Based on this finding, we delineated a putative mechanism implicating the modification of SK channels by cerebellar noradrenergic transmission in the pathway leading from stress to motor dysfunction.

INTRODUCTION

An intriguing feature of certain neurological disorders is the appearance of symptoms during episodes that punctuate an otherwise normal baseline phenotype. Episodic neurological disorders include several common conditions such as some forms of epilepsy, migraine, paralysis and movement disorders among others (Kullmann and Hanna, 2002; Ryan and Ptacek, 2010). While these may seem like a heterogeneous group, most result from mutations in ion channels and additionally share several features. First, despite the chronic expression of a defective ion channel, the baseline phenotype is unremarkable, with little or no sign of the disorder. Second, the symptoms are restricted to attacks, which can occur spontaneously or in response to certain stressors. Third, attacks of symptoms are induced by a common set of triggers that can broadly be classified as either chemical stressors, such as caffeine and ethanol or physical and psychological (Kullmann and Hanna, 2002; Ryan and Ptacek, 2010). It is frequently hypothesized that the existence of a shared set of triggers indicates that attacks are triggered by a mechanism common to all the triggers and similar across channelopathies. At the present, pinpointing the nature of the signaling pathways involved in the trigger-mediated response has been challenging, in part due to the fact that some of these triggers, such as caffeine and ethanol, under normal conditions have diametrically opposite effects on the nervous system. An effective strategy in the management of episodic disorders is to prevent the stressors from triggering the onset of symptoms; understanding their mode of action in the context of episodic dysfunction is therefore critical.

As a group triggers implicate a wide-variety of signaling pathways. One strategy to narrow down candidate mechanisms is to characterize the action of one and then examine the others within this context. To this end, we sought to delineate key components of the signaling pathways involved in the stress-mediated response in a well-established model of an episodic neurological disorder, the *tottering* mouse.

The most ubiquitous trigger across ion channelopathies involves some form of stress. Thus, either emotional or physical stress such as exercise triggers the onset of symptoms in paroxysmal dyskinesia, episodic ataxia and familial hemiplegic migraine among others (Richards and Barnett, 1968; Demirkiran and Jankovic, 1995; Battistini et al., 1999; Jen, 1999; Ryan and Ptacek, 2010). The physiological aspect of the stress response relies heavily on the noradrenergic system originating from the locus ceruleus in both humans and mice (Koob, 1999; Morilak et al., 2005). As such, the firing rates of locus ceruleus noradrenergic neurons increase when rats are exposed to stressful stimuli (Aston-Jones and Bloom, 1981; Abercrombie and Jacobs, 1987). The cerebellum is required for the expression of the *tottering*'s paroxysmal symptoms (Neychev et al., 2008), indicating that at least in this mouse trigger-induced changes ultimately converge onto this brain region. The noradrenergic afferents to the cerebellum originate exclusively from the locus ceruleus (Hokfelt and Fuxe, 1969; Bloom et al., 1971; Palay and Chan-Palay, 1974) and subjecting rats to restraint-stress leads to an increase in cerebellar norepinephrine levels (Saavedra et al., 1979). The noradrenergic system is therefore a likely candidate in the trigger pathway and in fact systemic blockade of alpha-noradrenergic receptors significantly decreases the frequency of stress-induced attacks in the *tottering* (Fureman and Hess, 2005).

In chapter 2 we found high frequency burst firing of Purkinje cells to be a hallmark of the *tottering*'s paroxysmal dyskinesia episodes. This characteristic Purkinje cell behavior provided the framework within which we investigated how stress forces a transition from normalcy to dysfunction. Moreover intracerebellar (IC) injections of NS309, an agent that increases the open probability of SK channels , abolished the attacks and regularized the Purkinje cell firing pattern. In principle triggers can induce Purkinje cells to burst through a wide variety of mechanisms without necessarily influencing the activity of SK channels. On the other hand, given the tight coupling of calcium current mediated by P/Q-type calcium channels to the activation of SK channels (Edgerton and Reinhart, 2003; Womack and Khodakhah, 2003), and the fact that P/Q-type calcium current density is already diminished in the *tottering* (Wakamori et al., 1998), the burst firing pattern associated with attacks *in vivo* can easily be mediated by a further decrease in the potassium current through SK channels. It has recently been shown that norepinephrine inhibits SK channel activity in dorsal root ganglia by activating a kinase that constitutively associates with and modulates the channel's gating properties (Maingret et al., 2008). Given the implication of noradrenergic signaling in the *tottering*'s attacks, we sought to evaluate this mechanism as a candidate pathway mediating the deleterious effects of stress on *tottering*'s motor function (Figure 1).

METHODS

Induction of attacks: Attacks were induced by restraining mice in a 60 mL syringe for 10 minutes, a commonly used paradigm to trigger attacks in the *tottering* (Fureman et al., 2002). Mice were videotaped 10 minutes prior to and for 40 minutes following restraint.

Systemic drug administration: Mice were transferred from their cage to the laboratory and each placed in a clean, empty cage and allowed to habituate for at least 2 hours prior to an injection. A total of 12 mice were used such that each mouse received one of two possible drugs and a total of 6 doses of the same drug on alternate days. A 10 ml/kg volume of each drug was injected subcutaneously. Table 1 provides a summary of all drugs and corresponding doses used as part of this study, their pharmacology as well as their presumed site of action. All systemically injected drugs were obtained from Tocris and the appropriate dose was prepared fresh every day. Clonidine and propranolol were prepared in 0.9% saline. Prazosin was prepared in 0.5% DMSO in 0.9% saline.

Intracerebellar injections: For intracerebellar injections, a single injection cannula (Plastics One) was stereotaxically implanted at midline (AP: -6.90 mm from Bregma and DV: 2 mm) under isfluorane anesthesia. Following recovery, a total volume of 3 μl of the desired solution was injected over a period of 15 minutes using an automated pump (World Precision Instruments). Cirazoline and norepinephrine (Sigma) were dissolved in 0.9% saline, solutions were made fresh every day by dissolving from a stock solution in DMSO.

Chronic perfusion of the cerebellum: We chronically perfused the cerebellum as previously described (Calderon et al., 2011). Under isofluorane anesthesia, double

cannula (Plastics One) was stereotaxically implanted at AP: -6.90 mm, ML: ± 1.5 mm from Bregma and DV: 2 mm and connected to an osmotic pump (0.25 $\mu l.h^{-1}$, Alzet). The pump was then placed under the skin on the back of the mice. Pumps were filled with solutions of 4,5,6,7-Tetrabromobenzotriazole (TBB, Tocris) made in 5% DMSO in 0.1% cyclodextrin and 0.01% methylene blue, which allowed for post-mortem examination of the perfusion site. The slow rate of perfusion is thought to allow the drug to remain contained within the cerebellum, with its concentration sharply dropping within 1 mm of the perfusion site (Calderon et al., 2011). Following surgery and 12 hours after, the long lasting NSAID pain reliever Flunixin was administered subcutaneously.

Disability rating scale: The severity of motor dysfunction was quantified according to a previously published scale (Weisz et al., 2005) as follows: 0=normal motor behavior, 1=slightly slowed or abnormal movements, 2=mild impairments, limited ambulation unless disturbed, 3=moderate impairment, limited ambulation even when disturbed, frequent abnormal postures, 4=severe impairment, almost no ambulation, sustained abnormal postures, 5=prolonged immobility in abnormal postures.

In vivo electrophysiology: Same procedure as described in Chapter 2. Throughout the recording session, mice were carefully monitored for the presence of an attack. The coefficient of variation of interspike intervals, predominant firing rate and mean firing rate of Purkinje and DCN neurons recorded during caffeine-induced attacks were obtained from the experiments performed as part of the study described in Chapter 2.

Optogenetic Stimulation of the locus ceruleus: 1.2 µl of virus (AAV2/1. CamKIIa.hChR2 (H134R)-EYFP; University of Pennsylvania Vector Core) was injected into the locus ceruleus bilaterally (AP: -5.8 mm; ML: ±1 mm; DV: -2.8 mm). Two optical

fibers (200 μm diameter, 0.48 NA, Thorlabs) were implanted to target the locus ceruleus. Light from a 473 nm or 450 nm laser (OEM Laser Systems) was delivered using fiberoptic ferrules (Kientec Systems) to the implanted fibers. Attenuation over the connector was measured before implantation to calculate output at the fiber tip during experiments. Stimuli were delivered as 10 ms light pulses at intensities ranging from <1 mW to 15 mW.

RESULTS

Noradrenergic transmission is required for stress to trigger attacks

We investigated whether noradrenergic transmission is necessary for stress to induce attacks using a pharmacological approach. In the first experiment, we blocked either β, α_1 or α_2 noradrenergic receptors systemically and assessed the frequency with which restraining the mice for 10 minutes, a well-established paradigm to induce attacks in the *tottering*, elicited attack (Fureman et al., 2002). We injected *tottering* mice with either 1 mg/kg prazosin, an α_1 antagonist (Cambridge et al., 1977), 0.1 mg/kg clonidine, an agonist of α_2 receptors (Cichini et al., 1986), 5 mg/kg propranolol, a β receptor antagonist (Atlas et al., 1974) or vehicle and 10 minutes later restrained them in a 60 mL syringe to induce stress. Doses for the drugs were selected prior to the start of the experiment according to dose ranges reported for mice and based on doses previously reported to inhibit attacks in the *tottering* (Fureman and Hess, 2005). In addition, we took into consideration the doses reported to influence locomotor behavior, 4 mg/kg for prazosin and 1 mg/kg for clonidine (Archer and Fredriksson, 2000; Mantsch et al., 2010), and ensured that the selected doses were significantly lower. We then confirmed that none of the injections caused sedation or any unusual behavioral effects in *tottering* mice. The presence or absence of a dyskinesia attack was determined by the experimenter. In a subset of experiments observers blinded to the conditions of the experiment determined the presence or absence of attacks by watching videos taken before and within 40 minutes following the injections.

In agreement with previous findings (Fureman and Hess, 2005), we found that systemic injection of 0.5 mg/kg of the α_1 adrenergic receptor antagonist prazosin

(Cambridge et al., 1977) 10 minutes prior to subjecting mice to restraint stress decreased the frequency with which restraint-stress induced attacks (mean frequency of restraint-induced attacks after pre-treatment with prazosin: 14 ± 9%, n = 6 trials each for N = 6 mice; vehicle: 75 ± 8%, n = 10 trials each in N = 6 mice; $p < 0.05$; Figure 2a). Similarly, 0.05 mg/kg clonidine, an agonist of α_2 receptors (Cichini et al., 1986), decreased the frequency of stress-induced episodes (mean percentage of restrained-induced attacks with clonidine pre-treatment: 7 ± 7%, n = 5 trials each in N = 6 mice; $p < 0.05$ relative to vehicle; Figure 2a). Conversely, pre-treating the mice with 5 mg/kg propranolol, a β-adrenergic receptor antagonist (Atlas et al., 1974) did not alter the susceptibility of the *tottering* to stress-induced attacks (mean percentage of restraint-induced attacks with propranolol pre-treatment: 70± 4%, n = 6 trials each in N = 6 mice; $p = 0.6$ relative to vehicle; Figure 2a).

There exists strong evidence indicating that the *tottering*'s paroxysmal symptoms are cerebellum and specifically Purkinje cell-dependent. For example, removing either abolishes attacks (Campbell et al., 1999; Neychev et al., 2008) and in addition, Purkinje cell specific expression of the *tottering* mutation recapitulates all of the mutant's symptoms (Mark et al., 2011). While ultimately the triggers' effects seem to converge on the cerebellum, their site of action is not known, and it is entirely possible that they act at upstream regions that provide the cerebellum with input. We reasoned that in the former case, injecting the triggers directly into the cerebellum should suffice to trigger an attack.

We performed intracerebellar injections (IC) of a total volume of 3 μL of either 3 μg caffeine, 10 μg norepinephrine or vehicle into *tottering*'s cerebellum over 15 minutes via a pre-implanted injection cannula and videotaped the mice for 40 minutes following injection to probe for the presence of attacks (Figure 2b). We found the IC injection of caffeine to significantly increase the frequency with which attacks occurred within the 40 minute time window following the injection (mean frequency of attacks after IC caffeine injection: 90 ± 7%, n = 10 trials each for N = 3 mice; vehicle: 15 ± 6%, n = 7 trials each in N = 3 mice; $p < 0.05$; Figure 2b). Similarly, IC injections of 10 μg norepinephrine reliably induced attacks (mean frequency of attacks after IC norepinephrine injection: 88 ± 6%, n = 9 trials each for N = 2 mice; $p < 0.05$ compared to vehicle; Figure 2b).

Stress-induced attacks are associated with erratic Purkinje cell activity

The hypothesized mechanism by which stress could trigger attacks relies on the finding that attacks associate with high frequency burst firing of Purkinje cells. In order to stably record the activity of single neurons in awake mice, our configuration requires us to head-restrain them with the aid of a surgically implanted head bracket. Since restraint is itself a paradigm used to stress the *tottering* mice (Fureman et al., 2002), it is not surprising that under these conditions mice exhibit frequent spontaneous attacks. Thus, we recorded the activity of Purkinje cells in the presence and absence of such spontaneous attacks, and compared this activity to that occurring with caffeine-induction to determine whether similar activity patterns associate with both triggers (Figure 3a).

When attacks occurred spontaneously presumably due to stress (referred to as spontaneous attacks in the text), we found the activity of *tottering* Purkinje cells to be

indistinguishable from that observed with caffeine injections. Spontaneous attacks were accompanied by a similar increase in the predominant firing rate (baseline: 74 ± 4 spikes/s; spontaneous attack: 170 ± 4 spikes/s; $p < 0.001$ relative to baseline, $p = 0.8$ relative to caffeine-triggered attacks; n = 11, N = 4; Figure 3b) and CV of ISIs (baseline: 0.66 ± 0.06; spontaneous attack: 1.47 ± 0.14; $p < 0.001$ relative to baseline; $p = 0.5$ relative to caffeine; Figure 3c), without a concurrent change in the mean firing rate (baseline: 52 ± 5 spikes/s; spontaneous attack: 56 ± 4 spikes/s; $p = 0.7$ relative to baseline; $p = 0.8$ relative to caffeine-triggered attacks; Figure 3d).

The firing of DCN neurons were similarly altered as well, with a predominant firing rate significantly above baseline during attacks (baseline: 63 ± 4 spikes/s, n = 20; N = 5; spontaneous attack: 93 ± 8 spikes/s, n = 23, N = 6; $p < 0.001$, caffeine attack: 88 ± 6 spikes/s, n = 21, N = 5; $p = 0.6$ relative to spontaneous attacks; Figure 3f) and ISI CV (baseline: 0.7 ± 0.05; spontaneous attack: 1.1 ± 0.1; $p < 0.001$; caffeine attack: 1.2 ± 0.1; $p = 0.3$ relative to spontaneous attacks; Figure 3g) without a concomitant change in the mean firing rate (Figure 3h).

In situ hybridization studies indicate that both α_1 and α_2 receptors reside within the cerebellum (Day et al., 1997). Noradrenergic α_1 receptors are located post-synaptically, where they transduce norepinephrine's effect on its target neuron by coupling to G protein (Civantos Calzada and Aleixandre de Artinano, 2001). On the other hand α_2 receptors are thought to reside mainly on the pre-synaptic side where they act as autoreceptors to inhibit the release of norepinephrine (Levin, 1984; Starke et al., 1989; Palij and Stamford, 1993), including in the cerebellum (Namima and Okamoto, 1987).

We sought to dissect the elements involved in norepinephrine's action on the cerebellum in three different ways. First, to determine whether blockade of noradrenergic receptors prevents attacks due to norepinephrine's action on the cerebellum, we performed IC injections of norepinephrine with or without pre-treatment of mice with systemic prazosin or clonidine prior to the onset of the IC injection. A total volume of 3 μL, 10 μg norepinephrine was injected into the cerebellum over 15 minutes, following pre-treatment with a systemic injection of prazosin, clonidine or vehicle. Given the presumed pharmacology of α_2 autoreceptors, if release of norepinephrine from pre-synaptic regions participates in the triggering mechanism, one would expect that systemically blocking these should have no effect on the frequency with which attacks are triggered when norepinephrine is injected into the cerebellum since this would presumably by-pass the need for endogenous norepinephrine to be released. On the other hand, if the released norepinephrine exerts its action through α_1 receptors, then systemically blocking these should alter the frequency with which attacks are triggered.

Indeed, with prazosin pre-treatment intracerebellar injections of norepinephrine did not elicit attacks as frequently as with vehicle pre-treatment (mean frequency of norepinephrine-induced attacks after pre-treatment with prazosin: 13 ± 7%, n = 4 trials each for N = 2 mice; vehicle: 88 ± 6%, n = 9 trials each in N = 2 mice; $p < 0.05$; Figure 4a). On the other hand, pre-treatment with clonidine had no effect on the ability of norepinephrine to elicit attacks (mean frequency of norepinephrine-induced attacks after pre-treatment with clonidine: 55 ± 6%, n = 4 trials each for N = 3 mice; $p = 0.1$ relative to vehicle; Figure 4a).

Conversely, to ensure it is the blockade of cerebellar α_1 adrenergic receptors that prevents stress from triggering attacks, we injected a total volume of 3 μL of 1.5 μg prazosin or vehicle into the cerebellum and immediately following the injection, restrained the mice in a 100 mL beaker for 10 minutes. We found the pre-treatment with IC injection of prazosin to decrease the frequency with which attacks occurred in response to restraint-stress (mean frequency of restraint-induced attacks after pre-treatment with IC prazosin: 15 ± 8%, n = 4 trials each for N = 3 mice; vehicle: 60 ± 8%, n = 7 trials each in N = 3 mice; $p < 0.05$; Figure 4a).

The third strategy we employed to substantiate the involvement of cerebellar α_1-receptors was to inject cirazoline, an α_1 agonist (Mottram and Saggar, 1985), into the cerebellum to assess whether this would suffice to induce attacks. IC injections of 3 μL, 2.5 μg cirazoline induced attacks significantly more frequently than injection of vehicle (mean frequency of attacks following IC cirazoline: 85 ± 8%, n = 5 trials each for N = 2 mice; vehicle: 5.8 ± 1%, n = 11 trials each in N = 3 mice; $p < 0.05$; Figure 4c). Together, these results suggest α_1 activation by norepinephrine is a likely candidate in the trigger mechanism employed by stress.

Release of norepinephrine from the locus ceruleus noradrenergic fibers innervating the cerebellum is sufficient to induce attacks

The firing rate of locus ceruleus neurons increases in response to various stressful stimuli (Aston-Jones and Bloom, 1981; Abercrombie and Jacobs, 1987). Moreover, electrical stimulation of the locus ceruleus has been shown to increase cerebellar norepinephrine content (Bickford-Wimer et al., 1991). We employed a similar strategy to promote the release of endogenous norepinephrine from the locus ceruleus using an

optogenetic approach. We stereotaxically injected an adeno-associated viral (AAV) vector containing channel rhodopsin (ChR2) into the locus ceruleus and implanted a fiber optic light guide to target the transfected neurons (Figure 5a). Strong optogenetic stimulations reliably produced attacks whereas control experiments with sham stimulations at non-functional wavelengths did not increase the frequency with which attacks occurred within the 120-minute time window the mice were observed (stimulation: n = 6 trials total in N = 3 mice; sham: n = 5 trials total in N = 3 mice; Figure 5 b, c, d). Interestingly, the latency of the stimulation-induced attack was similar to when triggers were used to induce them.

CK2-dependent phosphorylation of SK channels is a potential mechanism by which stress induces attacks.

It has previously been established that the post-natal Purkinje cell specific expression of the *tottering* mutation is sufficient to recapitulate its phenotype (Mark et al., 2011). SK channels play a crucial role in the maintenance of Purkinje cell excitability and blocking them results in burst firing *in vitro* (Womack and Khodakhah, 2003). Similarly, the burst firing observed *in vivo* could in principle result from a further decrease in the activity of SK channels. The intriguing finding that norepinephrine modulates SK channel gating properties so as to inhibit them (Maingret et al., 2008) led us to examine the involvement of this pathway in stress-induced attacks. The key kinase mediating norepinephrine's effect on SK channels was shown to be casein kinase 2 (CK2), a ubiquitous kinase that, along with calmodulin, constitutively associates with SK channels (Figure 1, Bildl et al., 2004). Upon activation, CK2 phosphorylates the SK

bound CaM and decreases the channel's calcium affinity five-fold (Figure 1, Allen et al., 2007). Thus, one way to assess the involvement of this pathway would be to chronically inhibit CK2 in the cerebellum of the *tottering* with the prediction that this should abolish attacks.

Independent observers blinded to the conditions of the experiment scored motor behavior before and after restraint stress and prior to, during and after chronic perfusion of the highly selective CK2 inhibitor 4,5,6,7-Tetrabromobenzotriazole (TBB) into the *tottering* cerebellum (Sarno et al., 2001; Pagano et al., 2008). Interestingly, 48 hours following the start of TBB perfusion, *tottering* mice stopped exhibiting attacks until the pumps depleted 14 days after their implantation (for each mice: n = 2 stress trials prior to start of perfusion; n = 6 stress trials during pump perfusion; n = 5 trials after pumps depleted starting 2 days after the end of perfusion; N = 3 mice; Figure 6).

DISCUSSION

A diverse set of symptoms is associated with paroxysmal episodes of ion channelopathies. Nevertheless, the triggers that precipitate the episodes are shared among the different mutations, indicating a common signaling pathway for attack initiation. We took advantage of the well-characterized and highly stereotypical attacks of dyskinesia in the *tottering* mouse model of EA2 to delineate the molecular underpinnings of episodes induced by stress, the most ubiquitous trigger among the paroxysmal movement disorders and channelopathies in general.

Pharmacologically blocking cerebellar α_1 receptors and preventing norepinephrine release by activating α_2 receptors resulted in a decrease in the frequency of restraint-induced attacks, whereas optogenetic stimulation of the locus ceruleus promoted their occurrence.

By recording from Purkinje cells in the awake *tottering* mouse under baseline conditions and during attacks triggered spontaneously, we found the same erratic activity to accompany stress and caffeine-induced episodes.

Given the evidence linking both SK channels and noradrenergic transmission in the trigger mechanism, the inhibition of SK channels by norepinephrine was investigated as a candidate pathway (Figure 1). Chronic cerebellar perfusion of TBB, a selective inhibitor (Sarno et al., 2001; Pagano et al., 2008) of a key kinase in the pathway mediating norepinephrine's effects (Maingret et al., 2008), alleviated *tottering*'s attacks. This effect remained for as long as TBB was perfused and disappeared upon cessation of the perfusion.

***Tottering* mice provide an invaluable tool for examining episodic motor dysfunction**

By virtue of the transiency of neuronal dysfunction, the substrates underlying the induction of attacks in channelopathies can only be delineated in the awake behaving animal. The presence of an animal model that can experimentally reproduce, in response to a similar set of triggers, the paroxysmal nature of the phenotype provided an invaluable opportunity to examine the signaling pathways underlying the episodic expression of symptoms.

The highly stereotypical nature of *tottering*'s dyskinesia attacks allows for reliable quantification of several parameters. First, doses of different stressors and the nature of the stress paradigm as well as the frequency with which a given dose or paradigm results in an attack have been quantified (Fureman and Hess, 2005). Second, the severity of the symptoms during attacks can reliably be measured using a published scale (Weisz et al., 2005; Alvina and Khodakhah, 2010a, b). Third, the time course of the attacks and the progression of the symptoms remain practically identical across attacks and mice (Shirley et al., 2008). The ability to reproducibly manipulate these parameters makes the *tottering* a unique mouse model to examine the paroxysmal basis of ion channel disorders.

On the other hand a substantial obstacle is the general lack of understanding of the dysfunctional circuitries that cause the paroxysmal symptoms (Kullmann et al., 2001). We relied on the finding that high frequency bursts of Purkinje cell firing accompany *tottering*'s attacks to scrutinize signaling pathways that would result in stress causing burst firing of Purkinje cells.

Noradrenergic transmission as a basis for paroxysmal dyskinesia

In the absence of a candidate mechanism we began our investigation using a pharmacological approach, which allows for rapid screening of different signaling pathways en masse. While this approach provided substantial evidence supporting our hypothesis, it is ultimately limited by the selectivity of the drugs employed to their targets at the doses needed to elicit effects *in vivo*. We were unable to perform each experiment using different drugs with similar presumed pharmacology due to the insolubility of high concentrations of virtually any noradrenergic agents other than the ones used as part of this study in solvents that would not be toxic to neurons. To compensate for this caveat, we sought to substantiate any evidence provided by eliciting it in a cerebellar-specific manner and blocking it in a cerebellar-specific manner as well.

We found the injection of either norepinephrine or the α_1 agonist cirazoline into the cerebellum to induce attacks. This result differs from previously published ones indicating that drugs expected to facilitate noradrenergic transmission do not increase the frequency of stress-induced attacks (Fureman and Hess, 2005). For example, it was found that neither the systemic injections of cirazoline nor that of desipramine, a drug that blocks the reuptake of norepinephrine, altered the frequency with which restraint induced attacks in the *tottering* (Fureman and Hess, 2005). Given the apparent blood brain barrier permeability of both these agents, this is a surprising finding and would likely require norepinephrine content to be measured in the cerebellum directly to be resolved. Norepinephrine, similarly to other catecholamines, cannot cross the *blood–brain barrier,* requiring the use of other drugs such as amphetamine to promote its increased release in

the brain (L'Heureux et al., 1986). In fact systemic amphetamine injections did not induce attacks in the *tottering* (Fureman and Hess, 2005) either. The advantage of intracerebellar injections as a complement to systemic injections is the ability to use norepinephrine itself to examine its effect. One explanation for the difference in the findings could be that a different receptor mediates norepinephrine's effects. *In situ* hybridization studies indicate that the noradrenergic receptor α_{1A} subtype is expressed in the cerebellum predominantly, with very little detection of mRNA for the α_{1B} and α_{1D} subtypes (Day et al., 1997). Cirazoline is a selective α_{1A} agonist with only partial activity at α_{1B} and α_{1D} subtypes whereas norepinephrine is an agonist of all three (Horie et al., 1995). While norepinephrine could in principle act at α_{1B} and α_{1D} receptors to elicit attacks, the virtual absence of mRNA for these subtypes would argue against this possibility. Examining the effect of alternative agents would help differentiate between these two possibilities and a genetic approach whereby the specific receptor subtype would be eliminated in the *tottering* cerebellum could resolve it.

Although the noradrenergic system is not directly affected by any of the mutations associated with episodic movement disorders, it has been implicated in their pathogenesis more than once. Our results concur with previous studies that found noradrenergic blockade to decreases stress-induced motor symptoms in several animal models of paroxysmal motor dysfunction. These include the *tottering* mouse, the *dt* rat, the dt^{sz} hamster and the Wriggle mouse Sagami (Richter and Loscher, 1998; Fureman et al., 2002). Although not all are channelopathies, these models show a stress-induced exacerbation of their respective motor symptoms as well, an effect abolished in the presence of noradrenergic blockers (Richter and Loscher, 1998).

Motor dysfunction as result of changes in K_{Ca} channel properties

The mechanism underlying stress-triggered attacks proposed here relies on a decrease in the SK channel current, which in Purkinje cells would ultimately result in a bursting pattern of firing (Womack and Khodakhah, 2003). SK channels essentially restrain the excitability of Purkinje cells in response to moderate increases in the intracellular calcium concentration and their pharmacological blockade induces burst firing (Edgerton and Reinhart, 2003; Womack and Khodakhah, 2003; Walter et al., 2006). Given the already diminished calcium current in the *tottering* (Wakamori et al., 1998), a decreased activation of SK channels is an attractive hypothesis in line with the burst firing pattern associated with attacks *in vivo*. That the attacks occur less frequently with oral administration of an SK channel activator (Alvina and Khodakhah, 2010a) and can be aborted via IC injection of a similar agent further supports such a hypothesis (Chapter 2). Several lines of evidence confirm the deleterious effect of decreases in K_{Ca} currents on motor dysfunction. For example, deletion of the *KCNN2* gene encoding the SK2 subunit results in severe ataxia and dyskinetic movement of the limbs in the *frissonnant* mouse (Szatanik et al., 2008). Similarly, mice lacking large-conductance calcium-activated potassium (BK) channels, exhibit severe ataxia and cerebellar infusion of BK channel blockers replicates this phenotype (Sausbier et al., 2004). In humans, mutations in the gene encoding BK channels are associated with the generalized epilepsy and paroxysmal dyskinesia syndrome (Du et al., 2005).

Norepinephrine modulation of SK channel properties

The gating properties of SK channels are a direct result of their affinity for calcium (Vergara et al., 1998). SK channels constitutively associate with calmodulin, which acts as the channel's calcium sensor (Vergara et al., 1998). When calcium binds to calmodulin the channel opens and then closes upon calcium unbinding (Vergara et al., 1998; Adelman et al., 2012). Additionally in complex with SK2 channels are CK2 and protein phosphatase 2A, the activation of which respectively increase and decrease their affinity for calcium by phosphorylating and dephosphorylating SK2-bound calmodulin (Bildl et al., 2004; Allen et al., 2007). By promoting the phosphorylation of the SK2-bound calmodulin by CK2 norepinephrine induces a decrease in the channel's affinity for calcium (Maingret et al., 2008). Interestingly, a substantial decrease in calmodulin expression was associated with a phenotype similar to that of the *tottering* mouse in the ataxic mouse pogo. Not surprisingly, pogo's Purkinje cells exhibit a bursting firing pattern (Lee et al., 2011). Our hypothesis and results are in agreement with the finding that norepinephrine's effect on SK channels is mediated by the α_1 subtype of noradrenergic receptors, as indicated by the fact that the effect is abolished in the presence of the α_1 receptor antagonist prazosin (Maingret et al., 2008).

A common pathway linking triggers of episodic neurological dysfunction

There are as yet very few clues with regard to the physiological mechanism underlying the triggers' action. The identification of the gene underlying paroxysmal non-kinesinegic dyskinesia (PNKD) as a key byproduct of caffeine and ethanol metabolism provided perhaps the first link between the triggers (Lee et al., 2012). The transgenic

PNKD mouse model is also reported to exhibit attacks of motor symptoms in response to caffeine and stress (Lee et al., 2012). At physiological doses, caffeine is an antagonist of adenosine receptors (Rainnie et al., 1994; Carter et al., 1995), which regulate the release of neurotransmitter at numerous synapses. It was recently shown that systemic injections of the A_{2A} adenosine receptor antagonist triggered attacks of dyskinesia in the PNKD mouse model (Lee et al., 2012). In our study, the induction of attacks in *tottering* mice was investigated using stress as a trigger and implicated an increased release of norepinephrine onto the cerebellum. Given that A_{2A} modulate the release of norepinephrine at various synapses (Moreau and Huber, 1999), examining whether the proposed mechanism involves an increased release of norepinephrine would help answer the interdependence of these two mechanisms.

Alternative hypotheses with regard to the triggers' effect in EA2 have relied upon the ability of various pharmacological agents to decrease the frequency of attacks. For example, it is thought that the expression of L-type calcium channels is upregulated in the *tottering* brain as compared to wild types (Campbell and Hess, 1999). Based on this observation, in one study, L-type calcium channel blockers were administered systemically and found to decrease the frequency of attack in *tottering* (Campbell and Hess, 1999). However, the Purkinje cell-specific expression of the *tottering* mutation is sufficient to recapitulate all of its motor symptoms (Mark et al., 2011). This suggests that triggers likely do not rely on developmental effects, such as the increased L-type channel expression, to mediate their effects.

The most parsimonious explanation with regard to the existence of a shared set of triggers that induce symptoms across a variety of channelopathies is that the underlying

trigger mechanism is shared as well. Given the variety of effects both caffeine and ethanol have on the nervous system, at which point in the induction pathway their effects converge is not immediately apparent. There are numerous pathways they can link them to norepinephrine, however at this point whether any of these play a role in the *tottering*'s attacks is a matter of speculation. As mentioned, at physiological doses caffeine is an antagonist of adenosinergic receptors and thus promotes the release of neurotransmitter, including that of norepinephrine (Rainnie et al., 1994; Carter et al., 1995). This provides a simple mechanism by which caffeine could modulate the release of norepinephrine. Given the diverse effects of ethanol, it is harder to postulate as to how it might result in such an increase. Nevertheless, several studies have found injections of physiological doses of ethanol to increase norepinephrine levels in the cerebellum (Lin et al., 1993).

A puzzling question with regard to the paroxysmal symptoms is that in humans, the episodes of neurologic dysfunction can last anywhere from minutes to several hours and typically outlast the pharmacological efficacy of ethanol, caffeine, or stress-induced changes in norepinephrine levels, which diminish far more rapidly compared to the time course of the symptoms. The basis for the mechanism proposed here is phosphorylation, a cellular process that operates on a time scale that is similar to that of the expression of symptoms. The molecular link coupling the activation of α_1 receptor to that of CK2 remains to be identified and would certainly help elucidate whether this mechanism participates in the induction of attacks by various triggers.

REFERENCES

Abercrombie ED, Jacobs BL (1987) Single-unit response of noradrenergic neurons in the locus coeruleus of freely moving cats. I. Acutely presented stressful and nonstressful stimuli. The Journal of neuroscience : the official journal of the Society for Neuroscience 7:2837-2843.

Adelman JP, Maylie J, Sah P (2012) Small-conductance Ca2+-activated K+ channels: form and function. Annual review of physiology 74:245-269.

Allen D, Fakler B, Maylie J, Adelman JP (2007) Organization and regulation of small conductance Ca2+-activated K+ channel multiprotein complexes. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:2369-2376.

Alvina K, Khodakhah K (2010a) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Alvina K, Khodakhah K (2010b) The therapeutic mode of action of 4-aminopyridine in cerebellar ataxia. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7258-7268.

Archer T, Fredriksson A (2000) Effects of clonidine and alpha-adrenoceptor antagonists on motor activity in DSP4-treated mice I: dose-, time- and parameter-dependency. Neurotoxicity research 1:235-247.

Aston-Jones G, Bloom FE (1981) Norepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. The Journal of neuroscience : the official journal of the Society for Neuroscience 1:887-900.

Atlas D, Steer ML, Levitzki A (1974) Stereospecific binding of propranolol and catecholamines to the beta-adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America 71:4246-4248.

Battistini S, Stenirri S, Piatti M, Gelfi C, Righetti PG, Rocchi R, Giannini F, Battistini N, Guazzi GC, Ferrari M, Carrera P (1999) A new CACNA1A gene mutation in acetazolamide-responsive familial hemiplegic migraine and ataxia. Neurology 53:38-43.

Bickford-Wimer P, Pang K, Rose GM, Gerhardt GA (1991) Electrically-evoked release of norepinephrine in the rat cerebellum: an in vivo electrochemical and electrophysiological study. Brain research 558:305-311.

Bildl W, Strassmaier T, Thurm H, Andersen J, Eble S, Oliver D, Knipper M, Mann M, Schulte U, Adelman JP, Fakler B (2004) Protein kinase CK2 is coassembled with small conductance Ca(2+)-activated K+ channels and regulates channel gating. Neuron 43:847-858.

Bloom FE, Hoffer BJ, Siggins GR (1971) Studies on norepinephrine-containing afferents to Purkinje cells of art cerebellum. I. Localization of the fibers and their synapses. Brain Res 25:501-521.

Calderon DP, Fremont R, Kraenzlin F, Khodakhah K (2011) The neural substrates of rapid-onset Dystonia-Parkinsonism. Nature neuroscience 14:357-365.

Cambridge D, Davey MJ, Massingham R (1977) Prazosin, a selective antagonist of post-synaptic alpha-adrenoceptors [proceedings]. British journal of pharmacology 59:514P-515P.

Campbell DB, Hess EJ (1999) L-type calcium channels contribute to the tottering mouse dystonic episodes. Molecular pharmacology 55:23-31.

Carter AJ, O'Connor WT, Carter MJ, Ungerstedt U (1995) Caffeine enhances acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors. The Journal of pharmacology and experimental therapeutics 273:637-642.

Cichini G, Lassmann H, Placheta P, Singer EA (1986) Effects of clonidine on the stimulation-evoked release of 3H-noradrenaline from superfused rat brain slices as a function of the biophase concentration. Temperature dependent widening of extracellular space. Naunyn-Schmiedeberg's archives of pharmacology 333:36-42.

Civantos Calzada B, Aleixandre de Artinano A (2001) Alpha-adrenoceptor subtypes. Pharmacological research : the official journal of the Italian Pharmacological Society 44:195-208.

Day HE, Campeau S, Watson SJ, Jr., Akil H (1997) Distribution of alpha 1a-, alpha 1b- and alpha 1d-adrenergic receptor mRNA in the rat brain and spinal cord. Journal of chemical neuroanatomy 13:115-139.

Demirkiran M, Jankovic J (1995) Paroxysmal dyskinesias: clinical features and classification. Annals of neurology 38:571-579.

Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Luders HO, Shi J, Cui J, Richerson GB, Wang QK (2005) Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. Nature genetics 37:733-738.

Edgerton JR, Reinhart PH (2003) Distinct contributions of small and large conductance Ca2+-activated K+ channels to rat Purkinje neuron function. JPhysiol 548:53-69.

Fureman BE, Hess EJ (2005) Noradrenergic blockade prevents attacks in a model of episodic dysfunction caused by a channelopathy. Neurobiology of disease 20:227-232.

Fureman BE, Jinnah HA, Hess EJ (2002) Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. Pharmacology, biochemistry, and behavior 73:631-637.

Hokfelt T, Fuxe K (1969) Cerebellar monoamine nerve terminals, a new type of afferent fibers to the cortex cerebelli. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 9:63-72.

Horie K, Obika K, Foglar R, Tsujimoto G (1995) Selectivity of the imidazoline alpha-adrenoceptor agonists (oxymetazoline and cirazoline) for human cloned alpha 1-adrenoceptor subtypes. British journal of pharmacology 116:1611-1618.

Jen J (1999) Calcium channelopathies in the central nervous system. CurrOpinNeurobiol 9:274-280.

Koob GF (1999) Corticotropin-releasing factor, norepinephrine, and stress. BiolPsychiatry 46:1167-1180.

Kullmann DM, Hanna MG (2002) Neurological disorders caused by inherited ion-channel mutations. Lancet Neurol 1:157-166.

Kullmann DM, Rea R, Spauschus A, Jouvenceau A (2001) The inherited episodic ataxias: how well do we understand the disease mechanisms? The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 7:80-88.

L'Heureux R, Dennis T, Curet O, Scatton B (1986) Measurement of endogenous noradrenaline release in the rat cerebral cortex in vivo by transcortical dialysis: effects of drugs affecting noradrenergic transmission. Journal of neurochemistry 46:1794-1801.

Lee HY, Nakayama J, Xu Y, Fan X, Karouani M, Shen Y, Pothos EN, Hess EJ, Fu YH, Edwards RH, Ptacek LJ (2012) Dopamine dysregulation in a mouse model of paroxysmal nonkinesigenic dyskinesia. The Journal of clinical investigation 122:507-518.

Lee KY, Kim JS, Kim SH, Park HS, Jeong YG, Lee NS, Kim DK (2011) Altered Purkinje cell responses and calmodulin expression in the spontaneously ataxic mouse, Pogo. The European journal of neuroscience 33:1493-1503.

Levin BE (1984) Axonal transport and presynaptic location of alpha 2-adrenoreceptors in locus coeruleus neurons. Brain research 321:180-182.

Lin AM, Bickford PC, Palmer MR, Gerhardt GA (1993) Ethanol inhibits the uptake of exogenous norepinephrine from the extracellular space of the rat cerebellum. NeurosciLett 164:71-75.

Maingret F, Coste B, Hao J, Giamarchi A, Allen D, Crest M, Litchfield DW, Adelman JP, Delmas P (2008) Neurotransmitter modulation of small-conductance Ca2+-activated K+ channels by regulation of Ca2+ gating. Neuron 59:439-449.

Mantsch JR, Weyer A, Vranjkovic O, Beyer CE, Baker DA, Caretta H (2010) Involvement of noradrenergic neurotransmission in the stress- but not cocaine-induced reinstatement of extinguished cocaine-induced conditioned place preference in mice: role for beta-2 adrenergic receptors. Neuropsychopharmacology 35:2165-2178.

Mark MD, Maejima T, Kuckelsberg D, Yoo JW, Hyde RA, Shah V, Gutierrez D, Moreno RL, Kruse W, Noebels JL, Herlitze S (2011) Delayed postnatal loss of P/Q-type calcium channels recapitulates the absence epilepsy, dyskinesia, and ataxia phenotypes of genomic Cacna1a mutations. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4311-4326.

Moreau JL, Huber G (1999) Central adenosine A(2A) receptors: an overview. Brain research Brain research reviews 31:65-82.

Morilak DA, Barrera G, Echevarria DJ, Garcia AS, Hernandez A, Ma S, Petre CO (2005) Role of brain norepinephrine in the behavioral response to stress. Progress in neuro-psychopharmacology & biological psychiatry 29:1214-1224.

Mottram DR, Saggar P (1985) Cirazoline, an alpha 2-adrenoceptor antagonist in guinea-pig ileum. General pharmacology 16:367-370.

Namima M, Okamoto K (1987) Modulatory action of prostaglandin D2 on the release of 3H-norepinephrine from rat cerebellar slices. Japanese journal of pharmacology 43:197-204.

Pagano MA, Bain J, Kazimierczuk Z, Sarno S, Ruzzene M, Di Maira G, Elliott M, Orzeszko A, Cozza G, Meggio F, Pinna LA (2008) The selectivity of inhibitors of protein kinase CK2: an update. The Biochemical journal 415:353-365.

Palay SL, Chan-Palay V (1974) Cerebellar Cortex: Cytology and Organization: Springer.

Palij P, Stamford JA (1993) Real-time monitoring of endogenous noradrenaline release in rat brain slices using fast cyclic voltammetry. 2. Operational characteristics of the alpha 2 autoreceptor in the bed nucleus of stria terminalis, pars ventralis. Brain research 607:134-140.

Rainnie DG, Grunze HC, McCarley RW, Greene RW (1994) Adenosine inhibition of mesopontine cholinergic neurons: implications for EEG arousal. Science 263:689-692.

Richards RN, Barnett HJ (1968) Paroxysmal dystonic choreoathetosis. A family study and review of the literature. Neurology 18:461-469.

Richter A, Loscher W (1998) Pathology of idiopathic dystonia: findings from genetic animal models. ProgNeurobiol 54:633-677.

Ryan DP, Ptacek LJ (2010) Episodic neurological channelopathies. Neuron 68:282-292.

Saavedra JM, Kvetnansky R, Kopin IJ (1979) Adrenaline, noradrenaline and dopamine levels in specific brain stem areas of acutely immobilized rats. Brain research 160:271-280.

Sarno S, Reddy H, Meggio F, Ruzzene M, Davies SP, Donella-Deana A, Shugar D, Pinna LA (2001) Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). FEBS Lett 496:44-48.

Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF, Ruth P (2004) Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. ProcNatlAcadSciUSA 101:9474-9478.

Shirley TL, Rao LM, Hess EJ, Jinnah HA (2008) Paroxysmal dyskinesias in mice. Movement disorders : official journal of the Movement Disorder Society 23:259-264.

Starke K, Gothert M, Kilbinger H (1989) Modulation of neurotransmitter release by presynaptic autoreceptors. Physiological reviews 69:864-989.

Szatanik M, Vibert N, Vassias I, Guenet JL, Eugene D, de Waele C, Jaubert J (2008) Behavioral effects of a deletion in Kcnn2, the gene encoding the SK2 subunit of small-conductance Ca2+-activated K+ channels. Neurogenetics 9:237-248.

Vergara C, Latorre R, Marrion NV, Adelman JP (1998) Calcium-activated potassium channels. CurrOpinNeurobiol 8:321-329.

Wakamori M, Yamazaki K, Matsunodaira H, Teramoto T, Tanaka I, Niidome T, Sawada K, Nishizawa Y, Sekiguchi N, Mori E, Mori Y, Imoto K (1998) Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. The Journal of biological chemistry 273:34857-34867.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. Nature neuroscience 9:389-397.

Weisz CJ, Raike RS, Soria-Jasso LE, Hess EJ (2005) Potassium channel blockers inhibit the triggers of attacks in the calcium channel mouse mutant tottering. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4141-4145.

Womack MD, Khodakhah K (2003) Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar Purkinje neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:2600-2607.

TABLE 1

Noradrenergic agents used as part of this study.

Drug	Systemic dose (mg/kg)	Intra-cerebellar dose (µg)	Pharmacology	Presumed site of action
Prazosin	0.5	1.5	antagonist	α_1-adrenergic receptor
Clonidine	0.05	0.8	agonist	α_2-adrenergic receptor
Propranolol	5	n/a	antagonist	β-adrenergic receptor
Cirazoline	n/a	2.5	agonist	α_1-adrenergic receptor
Norepinephrine	n/a	10	agonist	adrenergic receptor

FIGURES

FIGURE 1

Summary schematic of the proposed pathway for the induction of dyskinesia attacks in the *tottering* mouse by stress.

(a) Calcium entry through P/Q-type calcium channels increases the open probability of SK channels, which mediate an outward current. In response to norepinephrine, CK2 phosphorylates SK-bound calmodulin and decreases the channel's affinity for calcium 5-fold, causing Purkinje cells to burst (modified from Adelman et al., 2012).

(b) Characteristic firing pattern of a Purkinje cell in the presence (left) and absence of an attack.

(c) Stress induces dyskinesia attacks in the *tottering* mouse.

"NE": norepinephrine

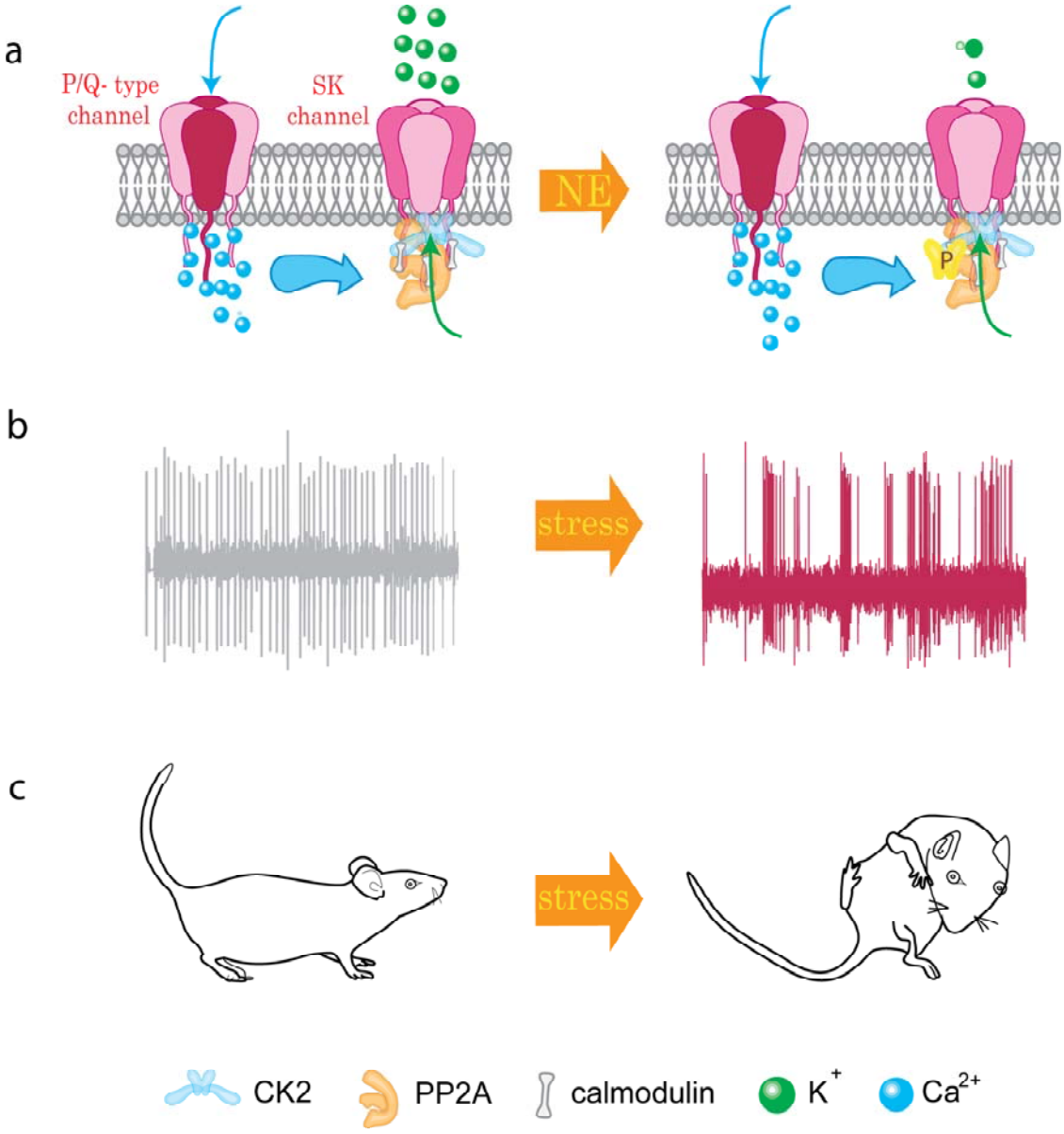

a
P/Q- type channel
SK channel
NE
P
b
stress
c
stress
CK2
PP2A
calmodulin
K+
Ca2+

FIGURE 2

Systemic and intracerebellar noradrenergic blockade prevents stress from triggering dyskinesia attacks.

(a) Compared to vehicle, systemic injection of the α_1 adrenoreceptor antagonist prazosin or α_2 adrenoreceptor agonist clonidine prior to subjecting *tottering* mice to restraint stress decreases the frequency with which restraint induces attacks. Injection of the β adrenoceptor antagonist propranolol does not alter the frequency of stress-induced attacks.

(b) Intracerebellar injections of either norepinephrine or caffeine induce attacks.

* denotes $p < 0.05$, IP: "intra-peritoneal", IC: "intra-cerebellar".

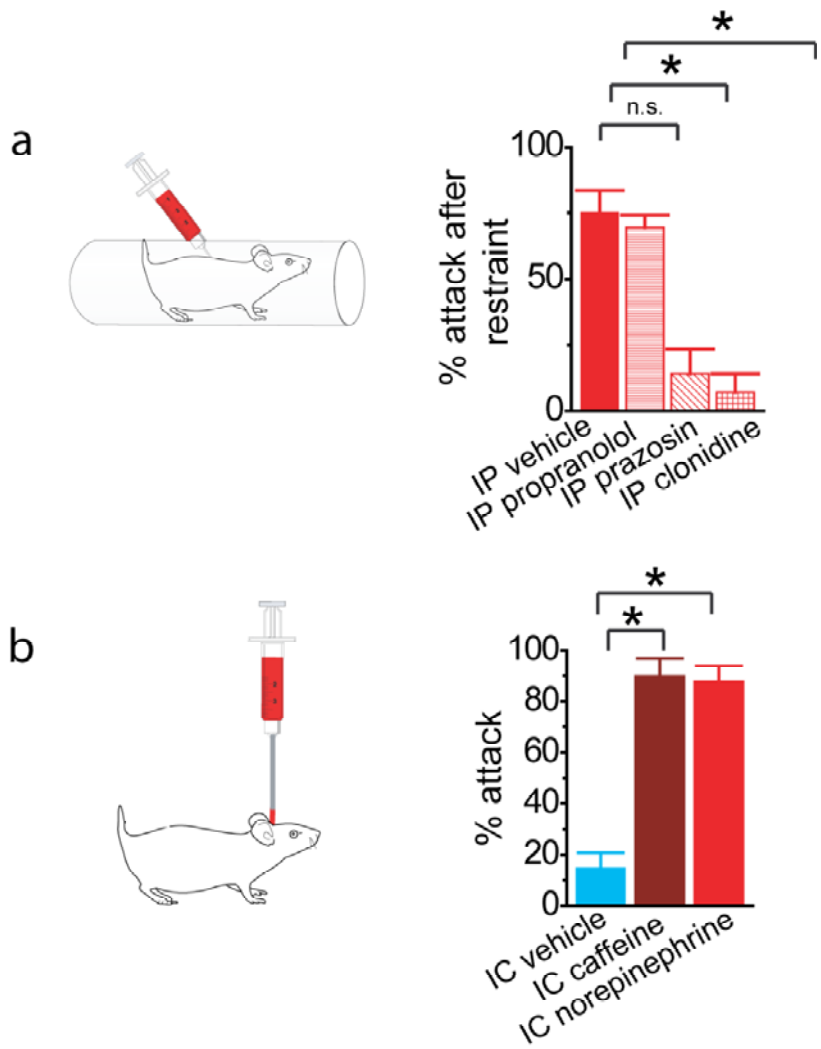

a
b
*
*
n.s.
100
50
0
% attack after restraint
IP vehicle
IP propranolol
IP prazosin
IP clonidine
*
*
100
80
60
40
20
0
% attack
IC vehicle
IC caffeine
IC norepinephrine

FIGURE 3

The firing pattern of *tottering* cerebellar neurons is similar during spontaneous and caffeine-triggered attacks.

(a) Purkinje cells were recorded in the awake head-restrained *tottering* mouse in the absence of an attack and when an attack was triggered either spontaneously or by subcutaneous caffeine injection.

(b,c) Similar increases in the predominant firing rate and ISI CV of *tottering* Purkinje cells occur when attacks trigger spontaneously *versus* in response to caffeine.

(d) Neither caffeine-triggered nor spontaneously occurring attacks associate with a change in the mean firing rate relative to baseline.

(e) DCN neurons were recorded in the awake head-restrained *tottering* mouse in the absence of an attack and when an attack was triggered either spontaneously or by subcutaneous caffeine injection.

(b, c, d, e) The firing characteristics of DCN neurons are similarly altered in the presence of spontaneous *versus* caffeine-triggered attacks.

* denotes $p < 0.05$, ** denotes $p < 0.001$.

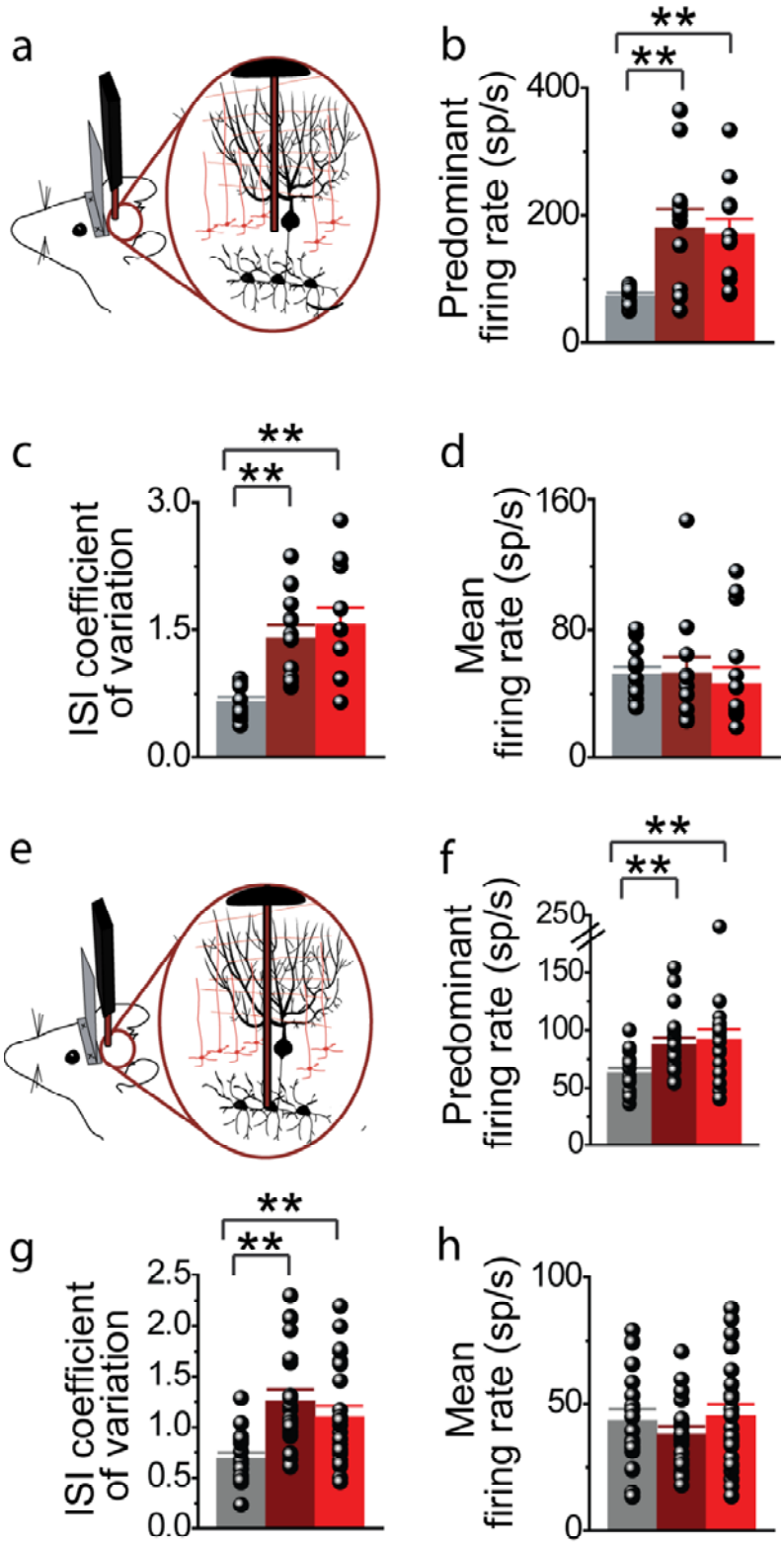

a
b
**
**
Predominant firing rate (sp/s)
400
200
0
c
**
**
ISI coefficient of variation
3.0
1.5
0.0
d
Mean firing rate (sp/s)
160
80
0
e
f
**
**
Predominant firing rate (sp/s)
250
150
100
50
0
g
**
**
ISI coefficient of variation
2.5
2.0
1.5
1.0
0.5
0.0
h
Mean firing rate (sp/s)
100
50
0

FIGURE 4

Intracerebellar injection of alpha noradrenergic agonists induce attacks.

(a) Systemically injecting *tottering* mice with the α_1 antagonist prazosin decreases the frequency with which attacks occur following intracerebellar injections of norepinephrine compared to pre-treatment with either vehicle or the α_2 agonist clonidine injections.

(b) Intracerebellar injection of prazosin decreases the frequency with which restraining *tottering* mice elicits attacks.

(c) The frequency of attacks following intracerebellar injection of the α_1 agonist cirazoline is significantly higher than that following vehicle injection.

* denotes $p < 0.05$, n.s. denotes "not significant".
IP: "intra-peritoneal", IC: "intra-cerebellar".

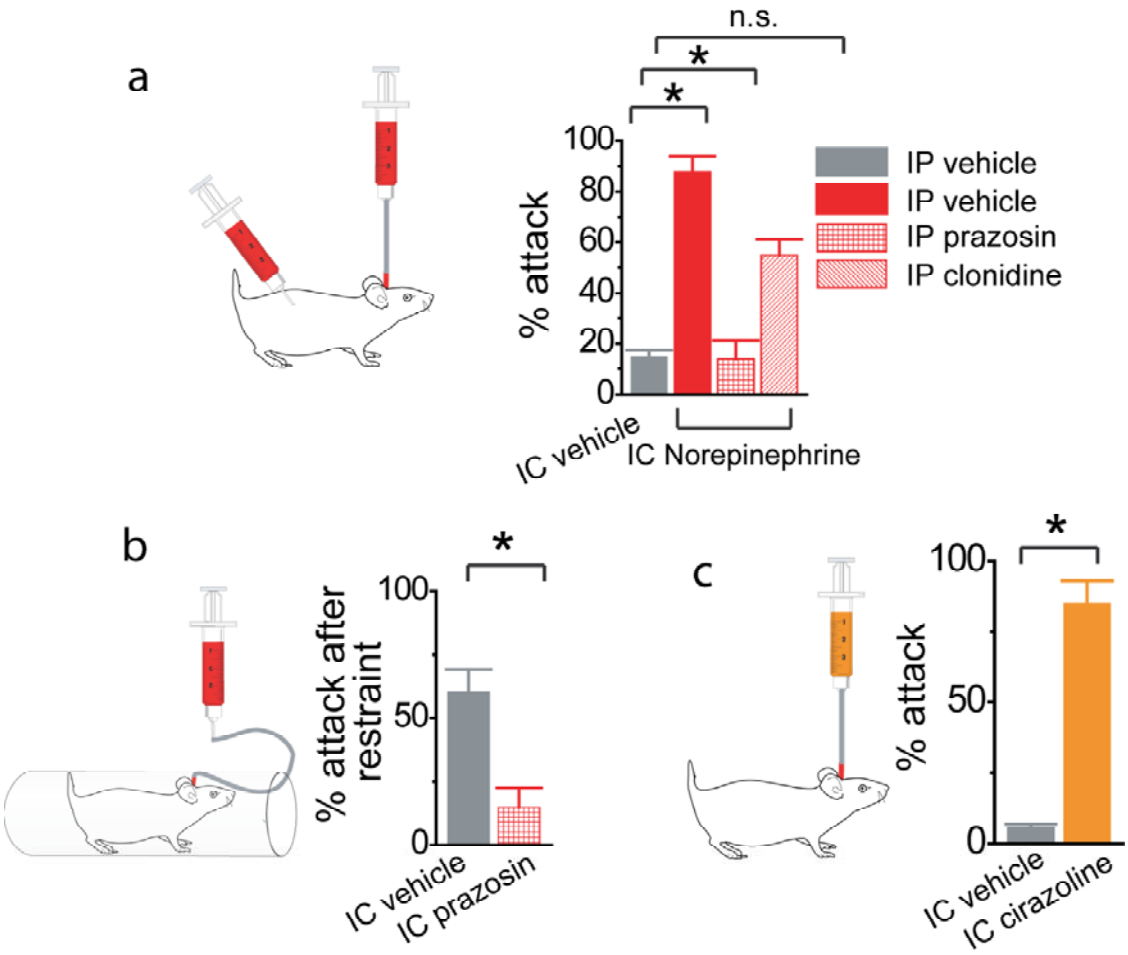

a
n.s.
*
*
100
80
60
40
20
0
% attack
IP vehicle
IP vehicle
IP prazosin
IP clonidine
IC vehicle
IC Norepinephrine
b
*
100
50
0
% attack after restraint
IC vehicle
IC prazosin
c
*
100
50
0
% attack
IC vehicle
IC cirazoline

FIGURE 5

Bilateral optogenetic stimulation of locus ceruleus triggers dyskinesia attacks in the *tottering*.

(a) Channel rhodopsin expression around and including the locus ceruleus visualized by GFP antibody staining and DAPI co-staining.

(b) *Tottering* mouse during optogenetic stimulation of the locus ceruleus.

(c) *Tottering* mouse during an attack elicited by optogenetically stimulating the locus ceruleus.

(d) The disability score is significantly higher following stimulation compared to sham stimulation at an inactive wavelength.

* denotes $p < 0.05$, "LC" denotes locus ceruleus.

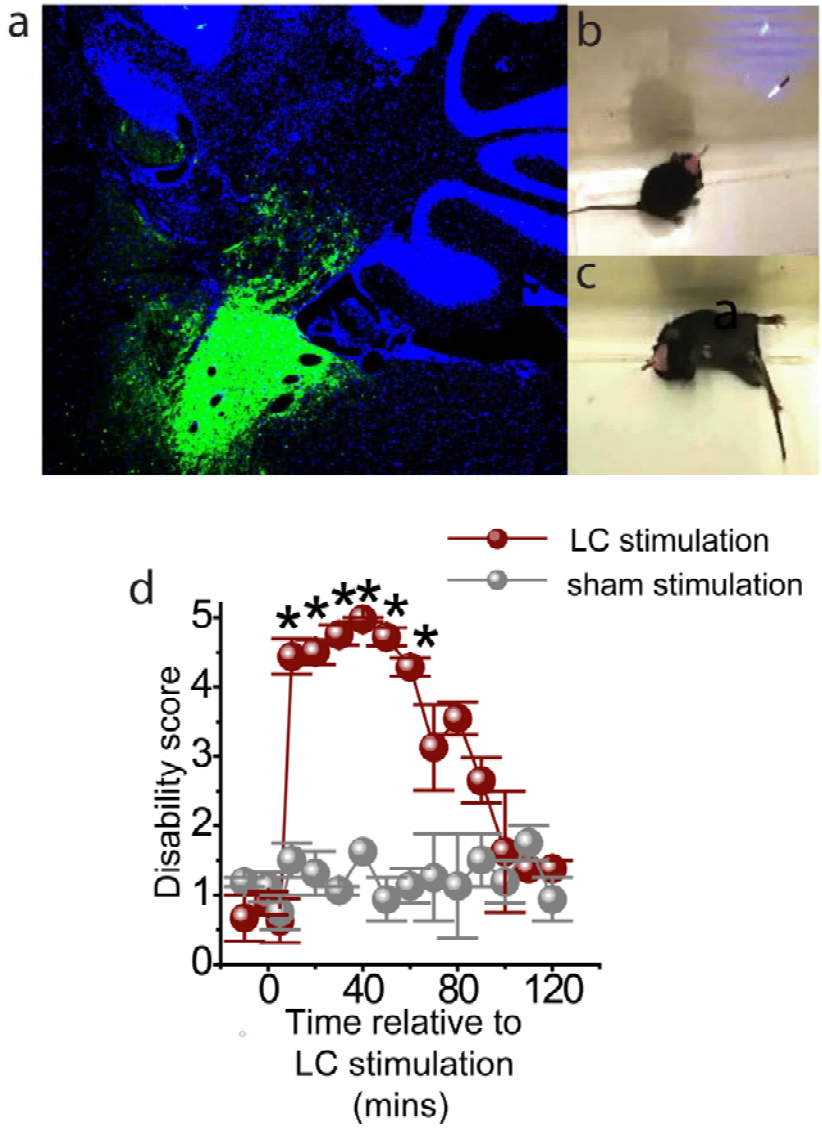
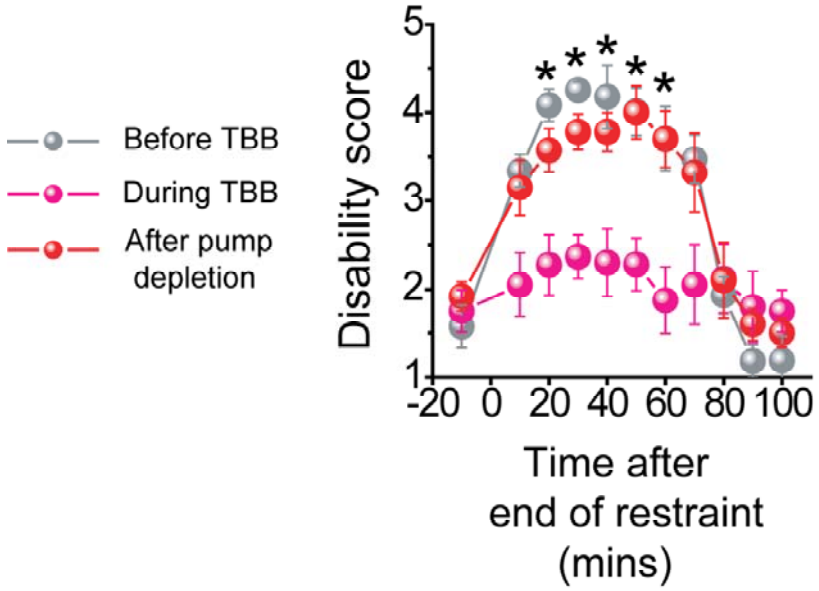

a
b
c
a
d
LC stimulation
sham stimulation
*****
Disability score
0
1
2
3
4
5
0
40
80
120
Time relative to
LC stimulation
(mins)

FIGURE 6

The frequency of stress-induced attacks is decreased during the chronic perfusion of a casein kinase II inhibitor into the cerebellum of the *tottering*.

The motor disability of the *tottering* scored after subjecting it to restraint-stress is significantly decreased during the perfusion of TBB compared to prior to the onset of perfusion and after the perfusion pumps deplete.

* denotes $p < 0.05$.


Before TBB
During TBB
After pump depletion
Disability score
5
4
3
2
1
-20
0
20
40
60
80
100
Time after end of restraint (mins)
*
*
*
*
*

CHAPTER IV:

CEREBELLAR PATHOPHYSIOLOGY OF A SPINOCEREBELLAR ATAXIA

Maria Johanna Dizon, Esra Tara[1], Joy T. Walter, Kellie A. Benzow,

Blake A. Ebner, Michael D. Koob, and Kamran Khodakhah

[1] Esra Tara contributed to figures 1, 4, 6 and 7

ABSTRACT

Spinocerebellar ataxia type 8 (SCA8) is a progressive movement disorder caused by a mutation in a noncoding RNA which is expected to perturb expression of Kelch-like 1 *(Klhl1)*. Both global and Purkinje cell-specific deletion of *Klhl1* in mice recapitulates the behavioral symptoms and anatomical pathology of SCA8, without altering cell number. This suggests that ataxia is caused by Purkinje cell dysfunction, and not cell death. Here we show both *in vivo* and *in vitro* that the targeted deletion of *Klhl1* in Purkinje cells results in a remarkable increase in synaptic inhibition onto Purkinje cells that caused their baseline firing rate to be lower and more erratic. This change likely reflects network compensation for an increase in Purkinje cell intrinsic excitability observed concomitantly. Restoring the precision of baseline Purkinje cell activity by oral administration of the K_{Ca} channel activator chlorzoxazone significantly improved motor performance in the knockout animals.

INTRODUCTION

The cerebellum integrates sensory and cortical information to coordinate voluntary movement and its dysfunction leads to poor limb coordination and balance, or *ataxia*, associated with a range of symptoms including unsteady gait, difficulty speaking, choking problems, and irregular eye movements (Ito, 1984). Cerebellar ataxia can be acquired or hereditary, with spinocerebellar ataxias (SCAs) being the most prevalent autosomal dominant hereditary ataxias, affecting 5-7/100,000 (Duenas et al., 2006). SCAs are a group of movement disorders that present with late-onset, slowly progressive ataxia accompanied by gradual atrophy of the cerebellum and related spinocerebellar pathways. So far, 31 different SCAs have been identified according to causative genetic locus (Schols et al., 2004; Soong and Paulson, 2007; Durr, 2010).

Spinocerebellar ataxia type 8 (SCA8) results from a CTG expansion in a noncoding RNA that overlaps the Kelch-like 1 (*Klhl1*) gene (Koob et al., 1999; Nemes et al., 2000), although it may also be associated with toxic byproducts of the expansion (Moseley et al., 2006). Klhl1 protein is mainly expressed in the brain, and enriched in cerebellar Purkinje cell somata and dendrites (Benzow and Koob, 2002; He et al., 2006). It displays homology with the Kelch family of actin-binding proteins first discovered in *Drosophila* (Robinson and Cooley, 1997), and their critical role in outgrowth of mammalian oligodendrocyte processes has recently been described (Jiang et al., 2007). However its function in mature mammalian neurons is unknown.

The global deletion of *Klhl1* in mice recapitulates the motor symptoms observed in SCA8 patients, namely late-onset slowly progressive gait abnormalities and cerebellar atrophy (He et al., 2006). In this mouse model, cerebellar atrophy relates solely to

thinning of the superficial molecular cell layer, but there is no other gross morphological change or loss of cerebellar neurons. Furthermore, when the deletion of *Klhl1* is restricted to Purkinje cells, the motor phenotype and cerebellar pathology are comparable to that observed in total-tissue deletions, pointing to a dysfunction specific to Purkinje cells as a major cause of ataxia (He et al., 2006). These Purkinje cell-specific *Klhl1* knockout mice are thus valuable targets of study not only for their potential relevance to SCA8, but because of insights that might be unearthed regarding the mechanisms underlying ataxia and cerebellar function, in general. To better understand the physiological defects that contribute to ataxia, we characterized cerebellar pathophysiology in these knockout mice.

The present work reveals two major defects in Purkinje cell function arising from deletion of *Klhl1*. We found, both *in vivo* and *in vitro*, an increase in inhibitory synaptic inputs onto Purkinje cells in the knockout animals that introduced significant noise in their baseline firing rate. In tandem, we observed an increase in the intrinsic excitability of the Purkinje cells themselves, which is likely the trigger for homeostatic network compensation in the form of increased interneuron activity. The mechanism underlying the aberrant Purkinje cell excitability can simply be accounted for by a change in the passive electrical properties of Purkinje cell dendrites as a consequence of *Klhl1* deletion. Finally, treating the knockout animals with the K_{Ca} channel activator chlorzoxazone to reduce the impact of increased interneuron activity on Purkinje cell baseline firing rate restored the firing characteristics of Purkinje cells and improved the motor performance of the mutant mice.

METHODS

Animals: Unless specified, experiments were performed using age-matched adult Pcp2-CRE/*Klhl1*$^{flox/flox}$ mice ("knockout, KO" mice, in which *Klhl1* was deleted specifically from Purkinje cells only) and *Klhl1*$^{flox/flox}$ mice ("wild-type, WT" littermates), at an age in which motor symptoms were fully evident. Descriptions of the generation and behavioral phenotype of these mice have been published previously (He et al., 2006).

To generate KO mice that expressed GFP selectively in Purkinje cells, P-Cre-3$^{+/+}$/*Klhl1*$^{flox/flox}$ homozygous mice were mated to a C57Bl6/J mouse to obtain offspring that were heterozygous at both the Cre transgene and the floxed *Klhl1* allele, and these offspring were crossed to obtain mice that were homozygous at the floxed *Klhl1* allele and heterozygous for the Cre transgene (P-Cre-3$^{+/-}$/*Klhl1*$^{flox/flox}$). These mice were bred with P-Cre-3$^{-/-}$/*Klhl1*$^{flox/flox}$ littermates to obtain the P-Cre-3$^{+/-}$/*Klhl1*$^{flox/flox}$ and P-Cre-3$^{-/-}$/*Klhl1*$^{flox/flox}$ sibling controls used in the experiments described. L7(PCP2)-GFP BAC transgenic mice that express GFP specifically in Purkinje cells (Gong et al., 2003) were bred with the P-Cre-3$^{+/-}$/*Klhl1*$^{flox/flox}$ mice and the offspring backcrossed to obtain the L7-GFP BAC/ P-Cre-3$^{+/-}$/*Klhl1*$^{flox/flox}$ and L7-GFP BAC/ P-Cre-3$^{-/-}$/*Klhl1*$^{flox/flox}$ sibling controls used in the Purkinje cell imaging studies.

Most experiments were performed blinded to the genotype. While the data reported were gathered using the Purkinje cell-specific mutant KO mice and their WT littermates, qualitatively comparable results were obtained from mice in which *Klkh1* was deleted globally. All experiments were performed in accordance with the guidelines set by Albert Einstein College of Medicine.

In vivo extracellular recording: Recordings were made from Purkinje cells, DCN neurons, and molecular layer interneurons of adult KO and WT mice under isofluorane anesthesia, and also from Purkinje cells in head-restrained awake adult KO and WT mice. To record, a 1.5 mm-diameter hole was drilled through the skull at 6 – 6.4 mm from Bregma and 1 to 2.5 mm from the midline. Extreme caution was taken to not touch the underlying cerebellar cortex. Signals from individual neurons were recorded extracellularly using platinum-quartz electrodes (2-3 MΩ; Thomas Recording GmbH) and a homemade differential amplifier. Purkinje cells were identified by depth, the presence of complex spikes, and distinct pauses in their firing after a complex spike. Molecular layer interneurons were identified by their depth, relative distance to the Purkinje cell layer, and the absence of complex spikes. Interneuron recordings were made from the most superficial layer of the cerebellar cortex to ensure that the electrode was positioned in the molecular layer. Moreover, the exact distance of the interneuron recording position from the first Purkinje cell layer was experimentally determined by advancing the electrode deeper to find the Purkinje cell layer to ensure that the electrode was appropriately positioned in the molecular layer. DCN neurons were identified on the basis of their location. In most experiments the recording location was marked by lesioning it by passing current through the recording electrode. The location of the lesion was ascertained by post-mortem slicing of the cerebellum.

Acute cerebellar slice preparation: Adult KO and WT mice were anesthetized with halothane and decapitated, and the brain was quickly removed and immersed in cold extracellular recording solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose (pH 7.4 with 5% CO_2-95% O_2) and the

cerebellum isolated. Sagittal (300 μm) slices were cut from the vermis of the cerebellum using a vibratome, allowed to recover in recording solution at 35°C (1 hr), and then maintained at room temperature until use (1-4 hr).

Single-unit extracellular recording in slices: Slices were mounted in a chamber on the stage of an upright Olympus Fluoview or Zeiss Axioskop with a 40× water-immersion objective (NA 0.8), and were superfused continuously at a rate of 1.5 ml/min with recording solution at 35 ±1°C. Extracellular recordings were made from individual Purkinje cells using a homemade differential amplifier. The tip of a glass pipette electrode (tip width same as patch pipettes, below) filled with recording solution was stably positioned near the Purkinje cell body at the axon hillock, where the largest voltage changes were usually recorded. Spontaneous action potentials were reported as negative deflections of 50-600 μV.

Whole-cell recording in slices: Patch electrodes were pulled from borosilicate glass with a resistance of 3-5 MΩ when filled with intracellular solution. Recordings were made at 35 ±1°C using an Optopatch amplifier (Cairn), with pipettes positioned on the soma. Purkinje cell IPSCs were recorded under whole-cell voltage clamp, with internal solution containing (in mM): 138 CsCl, 10 CsF, 10 HEPES, 3 MgATP, 2 QX-314, pH 7.2 with CsOH. The use of cesium allowed for a better space clamp and thus more accurate recording of IPSCs. With this high-chloride solution effecting symmetric external and internal chloride concentrations, GABAergic currents were inward when the cell was held at -60 mV. For these experiments 10 μM CNQX (Tocris) was included in the bath to block excitatory transmission. To record sIPSCs and mIPSCs, 3-5 minutes of activity were recorded per cell.

Purkinje cell EPSCs and EPSPs were recorded under whole-cell voltage and current clamp, respectively, with internal solution containing (in mM): 120 K- methyl sulfate, 10 KCl, 4 MgATP, 1.8 $MgCl_2$, 14 Na-creatine PO_4, 0.01 EGTA, 10 HEPES, 2 QX-314 (pH 7.2 with KOH); 100 μM picrotoxin (Sigma) and 1 μM CGP 55845 (Tocris) were included in the bath to block inhibitory synaptic transmission (Davies et al., 1993; Yoon et al., 1993; Davies, 2007). Purkinje cells were whole-cell voltage clamped at -60 mV and EPSCs were recorded in response to incrementally higher glutamate concentrations photoreleased over the Purkinje cell's dendrites. The protocol was repeated three times. Triplicate EPSPs were subsequently recorded under whole-cell current clamp in the same Purkinje cell in response to the same photolysis intensities. Cells were included in the analysis only if the series resistance was <12 MΩ and remained stable (within 20% change) throughout the recording. To estimate the dendritic membrane capacitance and input resistance current responses to voltage steps to –70 mV from a holding potential of –60 mV were fit with double exponential functions using Origin. The parameters obtained from this fit were used to determine the dendritic capacitance and resistance according to the methods outlined in Llano et al as previously described (Llano et al., 1991).

Electrical stimulation: The paired-pulse protocol was applied by delivering two 150 μs current pulses through glass electrodes (tip width same as patch pipettes) at an interval of 50 ms. Stimuli were targeted at the molecular layer interneurons around the Purkinje cell under study, and the intensity adjusted between 5-10 μA to elicit a clear response.

Photolytic stimulation: 250 μM MNI-caged-L-glutamate (Tocris) was pre-equilibrated into the slice in the recording chamber. Multi-line ultraviolet light from a continuous multi-line krypton ion laser (I302C, Coherent) was launched through a fiber optic cable into the epifluorescence port of the microscope, and focused onto the surface of the specimen plane through the same 40× water-immersion objective used to visualize the Purkinje cell under study. With the aid of a custom-written computer program, a 40 μm-diameter spot of UV light was directed over the dendrites of the target Purkinje cell to mimic activation of excitatory inputs. The duration (1 ms pulse) and intensity of the UV pulse was adjusted using an accousto-optical modulator (Neos). The range of stimulus intensities chosen was that which evokes Purkinje cell responses within physiological firing rates, as described previously (Walter and Khodakhah, 2006).

Purkinje cell dendrite volume estimates: Klh1 KO and WT mice expressing GFP selectively in Purkinje cells were imaged using a 100× objective with an Olympus Fluoview 1000 confocal laser-scanning microscope. The most distal portion of the molecular layer was selected for comparison (region of interest: 160 μm x 70 μm x 10 μm) and analyzed with Imaris software (Bitplane), using a "Surface Area Detail Level" of 0.25 μm.

Chlorzoxazone administration: During treatment phase, the animals' drinking water was replaced with 15 mM chlorzoxazone (Sigma) in 0.1% hydroxypropyl-β-cyclodextrin (Tocris) and 10% sucrose, as described previously (Alvina and Khodakhah, 2010). The solution was prepared fresh daily and administered to the animals via graduated 15 ml plastic tubes to facilitate measurement of intake. The animals' weight and water intake were recorded daily.

Accelerating rotarod paradigm: An animal was placed on a 3-cm-diameter rotating rod (Rotamex-5; Columbus Instruments) that was elevated 55 cm above a covered platform. The rod accelerated at 0.1 cm/s^2 until the animal fell off the rod. The animal's latency to fall was automatically recorded via Rotamex software. KO and WT mice were tested every day at the same time, during their dark cycle. The average latency of 10 consecutive trials was recorded.

Analysis: *In vitro* extracellular and whole-cell data were sampled at 10 and 20 kHz, respectively, using an A/D-D/A card (National Instruments) and PC (Dell), acquired and analyzed using LabView-based software written in-house. *In vivo* data was bandpass filtered (80 Hz – 500 Hz high pass, 10 kHz low pass) and sampled at 20 kHz. Waveforms were sorted using Offline Sorter software (Plexon Inc, Dallas, TX USA), and only those that could be unambiguously identified as belonging to a single unit were used in the analysis.

The predominant instantaneous baseline firing rate (FR) was determined by calculating the reciprocal of the predominant interspike interval (ISI) of the recording. Spontaneous and miniature IPSC events were detected and analyzed using Mini Analysis Program (Synaptosoft). Evoked IPSC, EPSC, and EPSP peak amplitudes were measured with respect to a set reference point at the baseline immediately before the stimulus. For paired-pulse IPSC recordings, the current peaks of 30 trials evoked at 1 Hz were averaged to calculate the paired-pulse ratio. For EPSCs and EPSPs, triplicate recordings at each photolysis intensity were first averaged within one cell, and then averaged across all cells.

Data are reported as means ± SEM, with P values calculated using one-way ANOVA or student's t-test as appropriate and considered statistically significant at $P<0.05$.

RESULTS

We directly examined whether deletion of *Klh1* in Purkinje cells altered their output by examining their firing rate *in vivo*. Extracellular recordings were made from individual Purkinje cells of adult Pcp2-CRE/*Klhl1*$^{flox/flox}$ (Purkinje cell-specific *Klh1* knockout, hereafter simply referred to as the KO) mice and *Klhl1*$^{flox/flox}$ (wild-type, WT) littermates (He et al., 2006) under isofluorane anesthesia (Figure 1A). We found that the mean predominant spontaneous firing rate of the KO Purkinje cells was a mere 47% of WT (KO= 26.9±3.4 spikes/s, n=30; WT= 51.4±5.1 spikes/s, n=24; P<0.00001). Moreover, the firing of KO Purkinje cells was considerably more erratic, as determined from the significantly higher coefficient of variation of interspike intervals (KO= 0.71±0.06; WT= 0.52±0.03, P=0.013). Activity of climbing fibers manifests as complex spikes in Purkinje cells. There was no difference in rate of complex spike activity in the WT or KO mice (KO= 1.01±0.06, n=12; WT= 1.07±0.06, n=8, P=0.44).

As expected from the lower inhibitory drive of Purkinje cells onto the neurons of the deep cerebellar nuclei (DCN) the firing rate of KO DCN neurons *in vivo* was significantly higher than that of the WT (Figure 1B; predominant firing rate: KO= 60.1±4.8 spikes/s, n=16; WT= 33.0±3.4 spikes/s, n=16; P<0.0001). Similarly to that seen in Purkinje cells, the firing of KO DCN neurons was also more erratic (coefficient of variation of interspike intervals: KO= 1.06±0.09, n=16; WT= 0.71±0.06, n=16; P=0.0018).

To explore the mechanisms responsible for the lower and more erratic firing rate of Purkinje cells in the KO mice we examined the activity of these neurons in acute cerebellar slices *in vitro*. Single-unit extracellular recordings from Purkinje cells in slices

(Figure 2A) also revealed a statistically significant lower predominant firing rate in the KO, although the difference was smaller (77%; KO= 47.6±2.1 spikes/s, n=36; WT= 61.6±2.9 spikes/s, n=50; P<0.001). The reduced firing rate of KO Purkinje cells *in vivo* and *in vitro* could be attributed to two possible mechanisms: 1) they either have a lower intrinsic firing rate, or 2) they receive stronger inhibitory synaptic transmission. Because in slice preparations synaptic circuitry is invariably compromised, the more erratic and lower firing rate of KO Purkinje cells seen *in vivo* compared with that seen in slices suggests that the latter possibility may be more compatible. Consistent with this idea, pharmacologically blocking synaptic inhibition in slices restored the KO Purkinje cell predominant firing rates to WT levels (Figure 2B; KO= 74.7±3.2 spikes/s, n=85; WT= 77.3±3.2 spikes/s, n=70; P=0.57), showing that the targeted deletion of *Klh1* in Purkinje cells significantly potentiates their inhibitory synaptic inputs. Conversely, neither the rate (KO= 90.1±6.5 spikes/s, n=10; WT= 78.2±7.5 spikes/s, n=17; P=0.289), nor precision of firing (KO CV= 0.089±0.010, n=10; WT CV= 0.084±0.013, n=17; P=0.796) of KO Purkinje cells was different after blocking fast excitatory and inhibitory synaptic transmission, indicating that the mutation does not affect intrinsic pacemaking (Walter et al., 2006).

In a limited number of animals available to us we also examined the firing rate of Purkinje cells in slices made from the cerebella of mice in which *Klhl1* was knocked out globally. We found that similar to that seen in Purkinje cell specific *Klhl1* KO mice the predominant firing rate of global knock out Purkinje cells were also lower than that of the wild type (general KO= 46.2±3.9 spikes/s, n=17; WT= 57.3±5.6 spikes/s, n=12). The heterozygous KO mice have an ataxic phenotype which is less severe than that of the

homozygous (He et al., 2006). As expected in these mice the predominant firing rate of heterozygous Purkinje cells was in between those of the WT and homozygous KO mice (53.1±3.3 spikes/s, n=18). Moreover, as seen in the Purkinje cell specific KO mice, the differences in the Purkinje cell firing rates of wild type and mutant mice were abolished when fast synaptic transmission was pharmacologically blocked (homozygous global KO= 74.0±7.1 spikes/s, n=25; heterozygous global KO= 72.9±4.5 spikes/s, n=25; WT= 78.9±7.2 spikes/s, n=6).

The increased synaptic inhibition in the mutant mice could result from changes in either the presynaptic GABAergic molecular layer interneurons, or from changes in the Purkinje cells themselves. The former might occur as a consequence of an increase in either the probability of GABA release from the interneurons, or in the spontaneous firing rate of interneurons. An increase in the number of synaptic GABA receptors on Purkinje cells, or alterations in the synapse that prolong the time course of GABA at the cleft, on the other hand, can also result in increased inhibition. Given that exactly the same observations were made in the Purkinje cell specific and global KO mice, we suspected one of the latter possibilities. To test these hypotheses, we recorded spontaneous inhibitory post-synaptic currents (sIPSCs) in Purkinje cells under whole-cell voltage clamp (Figure 3A). Much to our surprise, we found a substantial increase in the frequency of these events in the KO compared to WT (Figure 3B; KO= 19.0±3.1 Hz, n=11; WT= 10.0±1.7 Hz, n=14; P=0.013), but no appreciable difference in their mean amplitude (Figure 3C; KO= 44.3±3.4 pA, WT= 55.5±6.6 pA; P=0.177) or kinetics (Rise time KO= 4.0±0.29 ms, WT= 3.1±0.24 ms; P=0.028; Decay time KO= 8.7±0.59 ms, WT= 7.1±0.58 ms; P=0.089), suggesting a presynaptic origin. To further delineate the

underlying differences, we blocked spontaneous activity of interneurons by including TTX in the recording solution and examined the properties of miniature IPSCs (mIPSCs). Neither the frequency nor the amplitude of mIPSCs was different between KO and WT (Figures 3B and C), suggesting that the increased frequency of sIPSCs was caused by the higher firing rate of molecular layer interneurons rather than an increase in synaptic release probability.

To test the hypothesis that the firing rate of molecular layer interneurons was higher in the KO mice we measured their activity in anesthetized mice *in vivo* (Figure 4A). In remarkable agreement with the ≈2 fold higher frequency of sIPSCs recorded in KO cerebellar slices, the predominant firing rate of KO molecular layer interneurons *in vivo* was also significantly higher compared with those of the WT (KO= 66.1±8.3 spikes/s, n=6; WT= 24.9±3.2 spikes/s, n=12; $P<0.0001$). To test the hypothesis that the probability of neurotransmitter release at GABAergic synapses was not altered we examined the paired-pulse ratio of evoked IPSCs in voltage-clamped Purkinje cells in cerebellar slices. The mean paired-pulse ratio was the same in the KO and WT Purkinje cells (KO= 0.86±0.05, n=16; WT= 0.87±0.05, n=12; P=0.50, Figure 4B), in agreement with the notion that the release probabilities of GABAergic synapses were comparable. This finding also argues against the possibility of the involvement of retrograde messengers such as endocannabinoids in regulation of the firing rate of interneurons because these messengers typically affect release probability.

How could exclusive deletion of *Klhl1* selectively from Purkinje cells manifest with effects on the activity of interneurons presynaptic to them? We postulated that the increased inhibition could be the hallmark of a compensatory mechanism (Palop et al.,

2006) in response to Purkinje cell-specific defects. Such functional network adaptation might occur, for example, if *Klh1* deletion has somehow made Purkinje cells more excitable. To test this hypothesis we measured the input-output relationship of KO and WT Purkinje cells. With inhibition blocked, we first recorded excitatory post-synaptic-like currents (EPSCs) in a voltage-clamped Purkinje cell in response to increasing concentrations of glutamate briefly (1 ms) photoreleased over its dendrites. Then, excitatory post-synaptic potentials (EPSPs) in response to the same glutamate pulses were recorded in the same cell in current clamp configuration (Figures 5A and B). In agreement with our hypothesis, at each intensity we found that EPSCs in the KO Purkinje cells were, on average, ≈40% more efficacious in depolarizing them compared to the WT (n=5 WT, 4 KO; Figures 5C and D). Similarly, photorelease of a range of glutamate concentrations either directly on the dendrites of Purkinje cells or onto the underlying granule cells was ≈49% more efficacious in increasing the firing rate of KO Purkinje cells compared with that of the WT (n=6 WT, 5 KO; data not shown). The data presented therefore demonstrate that the KO Purkinje cells are more excitable than WT.

How could the loss of *Klhl1* increase the excitability of Purkinje cells? While deletion of *Klhl1* does not cause cell death or affect the gross anatomy of the cerebellum, it has been shown to decrease the thickness of the molecular layer of the cerebellar cortex which houses the dendrites of Purkinje cells by ≈20% (He et al., 2006). Although the function of *Klhl1* protein in Purkinje cells is unknown, its Kelch-family homologues are involved in cytoskeletal regulation (Adams et al., 2000). It is possible, therefore, that the thinning of the molecular layer in the *Klh1* KO is caused by smaller Purkinje cell dendrites mediated by defects in dendritic cytoskeletal regulation. However, although a

smaller dendritic surface area in the KO mice can arise from their thinner molecular layer (He et al., 2006), it is possible that the KO Purkinje cells are more morphologically compact. To test whether the dendritic surface area of the KO Purkinje cells is smaller, we estimated their dendritic capacitance (Llano et al., 1991) which is directly and linearly proportional to the surface area of dendrites. We found that the mean dendritic capacitance was ≈25% lower in the KO Purkinje cells compared with the WT (KO= 318.5±17.5 pF, n=19, WT= 426.3±17.0 pF, n=20; $P<0.05$, Figure 5E). Correspondingly, in remarkable agreement with the reduced dendritic surface area suggested by the 25% smaller dendritic capacitance, the dendritic input resistance was ≈22% higher in the KO Purkinje cells (KO= 146.6±14.8 MΩ, n=19, WT= 118.0±12.2 MΩ, n=20 $P<0.0001$, Figure 5E) although the somatic capacitance and input resistance were comparable between the two groups. The electrophysiological estimates of the size of the KO Purkinje cell dendrites, therefore, indicate the thinner molecular layer in the KO mice translate to smaller dendrites and argue against the possibility that the KO Purkinje cells dendrites are more compact. Nonetheless to directly examine whether the dendrites of KO Purkinje cells have the same density as WT or are more compact, we generated KO mice that expressed GFP selectively in Purkinje cells (Figure 5F) and measured their dendrite volume. We found that although the overall volume of the molecular layer was significantly decreased, the density of the KO Purkinje cell dendrites were comparable to those of the WT (Figure 5G). No difference was found in the Purkinje somatic volumes of KO vs. WT mice (KO: 1861±79 μm^3, WT: 1878±189 μm^3). Thus the morphometric data are in agreement with the electrophysiological estimates and suggest that the KO Purkinje cells have smaller dendrites. The reduced capacitance and the higher input

resistance of dendrites invariably dictate that the same excitatory synaptic input results in a greater depolarization. Thus, these findings suggest that the higher efficacy of EPSCs in depolarizing KO Purkinje cells is simply the consequence of the changes in passive electrical properties of Purkinje cells. While the efficacy of dendritic inhibitory inputs might also be similarly altered, the efficacy of the prominent somatic basket cell inhibitory inputs would be mostly unaffected.

It should be noted that while alterations in expression of voltage-gated channels (Aromolaran et al., 2007) might also in principle contribute to the greater excitability of Purkinje cells, the finding that the higher efficacy of EPSCs in the KO Purkinje cells did not depend on the magnitude of depolarization (Figure 5D) is in agreement with the proposition that changes in the dendritic properties are a major cause.

Collectively, the data presented are consistent with the hypothesis that the increased spontaneous activity of interneurons in the KO mice is a circuit level compensatory mechanism in an attempt to moderate the hyper-excitability of Purkinje cells to dendritic synaptic inputs that is caused by deletion of *Klhl1*. However, if the increase in the spontaneous activity of interneurons is a benevolent attempt to restore normal activity in Purkinje cells, then it is expected that the firing rate of KO Purkinje cells should be closer to those of the wild type in an awake mouse which presumably receives far greater excitatory inputs from cortico- and spinocerebellar mossy fibers than it does when it is anaesthetized. To test this hypothesis, we measured the firing rate of Purkinje cells in awake head-restrained mice. Remarkably, we found that in contrast to the two fold difference seen in the firing rate of Purkinje cells in anesthetized mice, in the awake mice as predicted the predominant firing rate of KO Purkinje cells was much

closer to that of the WT (KO: 51.3±3.8, WT: 69.1±11.4, n=20 and 9 respectively, p=0.07). Similarly to that seen in Purkinje cells, the firing of KO DCN neurons was also less altered in awake mice (KO: 52 ±4, WT: 48 ±7, n=22 and 16 respectively, p=0.6, Figure 6). These results are in agreement with the proposition that the increase in the spontaneous activity of molecular layer interneurons is a benevolent circuit-level compensation. So what is the cause of ataxia in the *Klh1* KO mice? One contributing factor to the ataxia seen in the *Klh1* KO mice might be the failure of homeostatic compensation in matching the firing rate of KO Purkinje cells with that of the WT under all behavioral conditions. Additionally, the increased rate of interneuron spontaneous activity in the KO mice inevitability introduces noise in the baseline activity of their Purkinje cells. A loss in the precision of Purkinje cell pacemaking reduces their ability to encode information and has been linked to ataxia (Hoebeek et al., 2005; Walter et al., 2006). If the noise introduced in the baseline activity of Purkinje cells by the increased spontaneous activity of interneurons makes a significant contribution to ataxia in the KO mice, then reducing their impact should lessen ataxia. Testing this hypothesis is challenging since there are no simple pharmacologic approaches that selectively target spontaneous interneuron firing or preferentially reduce the impact of increased spontaneous interneuron activity on Purkinje cells. Blocking GABA receptors *in vivo* to blocking inhibition, for example, would not only block the troublesome spontaneous interneuron inputs, but also all the driven interneuron inputs (i.e. feed-forward and lateral inhibition) that undoubtedly contain essential information. As an alternative approach, we sought to increase the precision of Purkinje cell baseline firing by taking advantage of the fact that under physiological conditions it is estimated that synaptically-driven feed-

forward or lateral inhibition is mediated by concurrent release of neurotransmitter from several (≈5) interneurons (Park et al., 2012), whereas spontaneous inputs correspond to neurotransmitter release from a single interneuron. We reasoned that it might be possible to increase the conductance of Purkinje cell action potential-locked calcium-activated potassium (K_{Ca}) channels to lessen the impact of spontaneous inhibitory inputs (from individual interneurons) on their pacemaking without preventing the ability of the stronger synaptically-evoked inhibition (arising from concurrent activity of multiple interneurons) in modulating their firing rate. One approach to increase action potential-locked K_{Ca} conductance is to increasing their affinity for binding calcium with compounds such as 1-EBIO and its analogues. Such K_{Ca} channel "activators" have been shown to restore precision of Purkinje cell firing in *ducky* and *tottering* mouse models of episodic ataxia type 2 by increasing the magnitude of the afterhyperpolarization between action potentials (Womack and Khodakhah, 2003; Walter et al., 2006; Alvina and Khodakhah, 2010). We thus supplemented the drinking water of the mice with chlorzoxazone (CHZ), a K_{Ca} channel activator that increases the precision of Purkinje cell pacemaking in *tottering* Purkinje cells and when given orally lessens their ataxia (Alvina and Khodakhah, 2010), and examined the baseline firing rate of Purkinje cells in anesthetized mice *in vivo*. Much to our delight, when the animals were treated with CHZ for 8 days, we found that the erratic firing of Purkinje cells of KO mice was significantly reduced and had become indistinguishable from WT (Figure 6A; coefficient of variation of interspike intervals: KO= 0.81±0.1; KO+CHZ= 0.56±0.03; WT= 0.52±0.03; KO+CHZ vs. KO, P=0.026; KO vs. WT, P=0.003). To test if such a decrease in the baseline Purkinje cell erratic activity at the cellular level would translate to a reduction in ataxia at

the behavioral level, the motor performance of WT and KO animals was assayed daily using an accelerating rotarod paradigm before, during, and after oral administration of 15 mM CHZ (Figure 6B and C). Animals were trained to proficiency for 10 days and allowed to plateau for 12 days, treated with CHZ for 15 days, and returned to normal drinking water for 10 days. Remarkably, treatment with the same concentration of CHZ that reduced the baseline Purkinje cell erratic activity improved the motor performance of KO animals without affecting that of the WT (mean latency to fall: KO+CHZ= 59.5±0.7 s, KO untreated= 51.6±0.5 s, $P<0.0001$, n=4; WT+CHZ= 68.2±0.8 s, WT untreated= 70.6±1.2s, $P=0.087$, n=6; Figures 6B and C).

Collectively, the data presented support the hypothesis that the erratic baseline activity of Purkinje cells in the KO mice contributes to the ataxia seen in these animals.

DISCUSSION

We took advantage of a mouse model of SCA8 to examine the pathophysiology of this disorder. In these mice the observed motor deficits are attributable to Purkinje cell dysfunction because the mice do not exhibit any cerebellar cell loss, and deletion of Kelch-like 1 protein is restricted to Purkinje cells. Our study revealed two main defects. The mutant Purkinje cells exhibited an atypically lower and more erratic firing rate which arose from an increase in the rate of spontaneous activity of GABAergic molecular layer interneurons. The mutant Purkinje cells were also more excitable. The increased excitability of knockout Purkinje cells can be accounted for by the changes in their passive electrical membrane properties as a direct consequence of their smaller dendrites caused by deletion of *Klhl1*. The enhanced synaptic inhibition, therefore, appears to be a homeostatic network-level compensation in response to a Purkinje cell-specific dysfunction, namely a significant elevation in their intrinsic excitability. While largely beneficial, an unavoidable side effect of the increased spontaneous activity of interneurons is the introduction of significant noise in the baseline activity of Purkinje cells that deteriorates their ability to encode information (Walter et al., 2006) and contributes to ataxia in *Klhl1* KO mice.

Degeneration vs. dysfunction: utility of the Klhl1 knockout mouse model

Mice in which *Klhl1* was deleted globally, or specifically in Purkinje cells displayed comparable motor deficits and histological abnormalities (He et al., 2006) and pathophysiology. Such persistence in phenotype at multiple levels reinforces the assertion that Purkinje cells are the specific substrate of the ataxia observed in these mice. Importantly, the absence of cell death implies that not only are Purkinje cells involved,

but that it is specifically their dysfunction and not their reduction in number that directly underlies the symptoms. This is not the first description of dysfunction, as opposed to degeneration, being the fundamental causative agent of a progressive disorder: SCA1 and *ataxia* mice are ataxic before neuronal loss is detected (Clark et al., 1997; Wilson et al., 2002; Shakkottai et al., 2004), and motor and cognitive symptoms precede cell death in a few mouse models of Huntington's disease (Joshi et al., 2009). However, to our knowledge, the *Klhl1* knockout mouse is the only model that faithfully mimics symptom progression of SCA8 without ever exhibiting any pathological cell loss. While this also substantiates the likelihood that SCA8 is a *Klhl1* loss-of-function disease, whether SCA8 is caused by loss of *Klhl1* or accumulation of toxic by-products of the CTG expansion deserves further scrutiny (Moseley et al., 2006). Nonetheless, in addition to providing information regarding factors that lead to cerebellar dysfunction in general, the Purkinje cell-specific *Klhl1* knockout mouse model is a valuable tool for studying how specific alterations in Purkinje cells can lead to progressive ataxia, including SCA8.

Klhl1, cytoskeletal regulation, and motor coordination

Klhl1 is so named for its structural homology with the *Kelch* superfamily, first described to bind actin during oogenesis in *Drosophila* (Robinson and Cooley, 1997). While the specific function of *Klhl1* in mature mammalian neurons is unknown, its general role in the stabilization, arrangement, and plasticity of the actin-myosin cytoskeleton is now increasingly clear. For example, it has recently been shown to promote process elongation of oligodendrocytes necessary for proper myelination (Jiang et al., 2007). The present work is the first demonstration of the effects of *Klhl1* deletion in mature mammalian neurons. Specifically, the data presented show that loss of *Klhl1* in

Purkinje cells results in their having smaller dendrites, and that the decrease in the dendritic surface area results in changes in the passive properties of Purkinje cells such that they become hyper-excitable. Intriguingly, a growing number of reports associate disruptions of cytoskeletal organization and machinery with progressive diseases, including progressive ataxia. Alterations in function of Rac GTPases, which play a role in structuring the cytoskeleton, are associated with progressive cerebellar ataxia (Luo et al., 1996). Similarly, deletion of one of its activators, P-Rex2, a Rac-guanine-nucleotide exchange factor (Rac-GEF) that is highly expressed in Purkinje cells, leads to progressive alterations in Purkinje cell dendritic length and morphology and concurrently worsening ataxia in mice (Donald et al., 2008).

Compensation for dysfunction and other inherited ataxias

As discussed above, the smaller dendritic tree of Purkinje cells and thus their increased excitability in *Klhl1* KO is consistent with the known functions of this family of proteins. Given that in the Purkinje cell-specific *Klhl1* KO mice we also observed a marked increase in the activity of molecular layer interneurons, the most parsimonious interpretation is likely that the latter is network compensation in an attempt to maintain homeostasis. In fact, increasing the frequency of the spontaneous firing of interneurons in principle makes the most effective use of GABAergic inputs to reduce the input resistance of Purkinje cells to render them less excitable. Such a compensatory mechanism would be superior than increasing release probability at GABAergic synapses, increasing GABA receptor density, or even prolonging the time course of individual IPSCs. This is because all of these alternatives would also affect the efficacy of feed-forward and lateral inhibition. A down side of increasing spontaneous inhibitory

drive to lessen the excitability of Purkinje cells, however, is that it makes their baseline activity more erratic. As evidenced by the pharmacological rescue of the precision of Purkinje cell firing and the concurrent motor improvement, the erratic firing of Purkinje cell activity in *Klhl1* KO mice appears to be a major contributing factor to ataxia in these mice.

The fact that the genetic defect originates in the Purkinje cells makes it highly improbable that the reverse is true, namely that Purkinje cells are more excitable as compensation for an increased GABAergic drive. However, our data do not rule out this or additional disturbances working in conjunction to sculpt the motor symptoms experienced by ataxic patients. It is nevertheless striking that the physiological defects identified in this study are paralleled in other models of inherited ataxia. For example, increased inhibition and excitability of Purkinje cells have been documented in mouse models of episodic ataxia type 1 (EA1) (Zuberi et al., 1999; Herson et al., 2003), and erratic firing of Purkinje cells has been observed in both EA1 and EA2 models (Walter et al., 2006; Davies, 2007).

It would seem that erratic cerebellar output is a common theme amongst many different types of ataxia. The function of Purkinje cells is to convey the sole output of the cerebellar cortex by processing over 150,000 excitatory and inhibitory inputs on top of an intrinsic baseline (Ito, 1984; Hoebeek et al., 2005; Walter and Khodakhah, 2009). The commonalities in these disorders imply that, ultimately, any mechanism that sufficiently degrades the signal-to-noise ratio or limits the contents of the encoded information will lead to ataxia. In keeping with this idea, in the model of SCA8 examined here improving

the precision of baseline firing of KO Purkinje cells with chlorzoxazone improved the motor performance of KO animals.

Both in SCA8 patients and in our *Klhl1* KO model of this disorder ataxia is progressive and appears late in life. Normal neuronal cell death and degeneration associated with aging has been implicated in decreased motor coordination (Forster et al., 1996). In the case of progressive ataxias, these effects would compound when genetic dysfunction and age-induced degeneration combine. Such impact would be maximized by the absence of successful compensatory machinery. The differential failure of compensatory mechanisms may help subserve variations in the expression and progression of symptoms.

The present work also highlights the intricacies of CNS function. There are numerous examples of homeostatic mechanisms which successfully maintain proper physiologic function in the face of diverse challenges (Palop et al., 2006). The data presented demonstrate that in the case of *Klh1* KO mice a subtle change in the size and thus excitability of Purkinje cells sets in motion compensatory mechanisms that, while largely beneficial, ultimately fail to fully restore normalcy to the circuit.

REFERENCES

Adams J, Kelso R, Cooley L (2000) The kelch repeat superfamily of proteins: propellers of cell function. Trends in cell biology 10:17-24.

Alvina K, Khodakhah K (2010) KCa channels as therapeutic targets in episodic ataxia type-2. JNeurosci 30:7249-7257.

Aromolaran KA, Benzow KA, Koob MD, Piedras-Renteria ES (2007) The Kelch-like protein 1 modulates P/Q-type calcium current density. Neuroscience 145:841-850.
Benzow KA, Koob MD (2002) The KLHL1-antisense transcript (KLHL1AS) is evolutionarily conserved. Mammalian genome : official journal of the International Mammalian Genome Society 13:134-141.

Clark HB, Burright EN, Yunis WS, Larson S, Wilcox C, Hartman B, Matilla A, Zoghbi HY, Orr HT (1997) Purkinje cell expression of a mutant allele of SCA1 in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:7385-7395.

Davies CH, Pozza MF, Collingridge GL (1993) CGP 55845A: a potent antagonist of GABAB receptors in the CA1 region of rat hippocampus. Neuropharmacology 32:1071-1073.

Davies F, Adelman, J.P., and Maylie, J. G. (2007) Cerebellar Purkinje cells in a mouse model of episodic ataxia/myokemia type1 display reduced precision of tonic firing mediated by GABAergic input from cerebellar interneurons. . In: Society for Neuroscience, 2007 San Diego, CA.

Donald S, Humby T, Fyfe I, Segonds-Pichon A, Walker SA, Andrews SR, Coadwell WJ, Emson P, Wilkinson LS, Welch HC (2008) P-Rex2 regulates Purkinje cell dendrite morphology and motor coordination. Proceedings of the National Academy of Sciences of the United States of America 105:4483-4488.

Duenas AM, Goold R, Giunti P (2006) Molecular pathogenesis of spinocerebellar ataxias. Brain : a journal of neurology 129:1357-1370.

Durr A (2010) Autosomal dominant cerebellar ataxias: polyglutamine expansions and beyond. Lancet Neurol 9:885-894.

Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS (1996) Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proceedings of the National Academy of Sciences of the United States of America 93:4765-4769.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, Heintz N (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature 425:917-925.

He Y, Zu T, Benzow KA, Orr HT, Clark HB, Koob MD (2006) Targeted deletion of a single Sca8 ataxia locus allele in mice causes abnormal gait, progressive loss of motor coordination, and Purkinje cell dendritic deficits. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:9975-9982.

Herson PS, Virk M, Rustay NR, Bond CT, Crabbe JC, Adelman JP, Maylie J (2003) A mouse model of episodic ataxia type-1. NatNeurosci 6:378-383.

Hoebeek FE, Stahl JS, van Alphen AM, Schonewille M, Luo C, Rutteman M, van den Maagdenberg AM, Molenaar PC, Goossens HH, Frens MA, De Zeeuw CI (2005) Increased noise level of purkinje cell activities minimizes impact of their modulation during sensorimotor control. Neuron 45:953-965.

Ito M (1984) The Cerebellum and Neural Control. New York: Raven.
Jiang S, Seng S, Avraham HK, Fu Y, Avraham S (2007) Process elongation of oligodendrocytes is promoted by the Kelch-related protein MRP2/KLHL1. The Journal of biological chemistry 282:12319-12329.

Joshi PR, Wu NP, Andre VM, Cummings DM, Cepeda C, Joyce JA, Carroll JB, Leavitt BR, Hayden MR, Levine MS, Bamford NS (2009) Age-dependent alterations of corticostriatal activity in the YAC128 mouse model of Huntington disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:2414-2427.

Koob MD, Moseley ML, Schut LJ, Benzow KA, Bird TD, Day JW, Ranum LP (1999) An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8). Nature genetics 21:379-384.

Llano I, Marty A, Armstrong CM, Konnerth A (1991) Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. JPhysiol 434:183-213.
Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 379:837-840.

Moseley ML, Zu T, Ikeda Y, Gao W, Mosemiller AK, Daughters RS, Chen G, Weatherspoon MR, Clark HB, Ebner TJ, Day JW, Ranum LP (2006) Bidirectional expression of CUG and CAG expansion transcripts and intranuclear polyglutamine inclusions in spinocerebellar ataxia type 8. Nature genetics 38:758-769.

Nemes JP, Benzow KA, Moseley ML, Ranum LP, Koob MD (2000) The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1). Human molecular genetics 9:1543-1551.

Palop JJ, Chin J, Mucke L (2006) A network dysfunction perspective on neurodegenerative diseases. Nature 443:768-773.

Park SM, Tara E, Khodakhah K (2012) Efficient Generation of Reciprocal Signals by Inhibition. Journal of neurophysiology.

Robinson DN, Cooley L (1997) Drosophila kelch is an oligomeric ring canal actin organizer. The Journal of cell biology 138:799-810.

Schols L, Bauer P, Schmidt T, Schulte T, Riess O (2004) Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis. Lancet neurology 3:291-304.

Shakkottai VG, Chou CH, Oddo S, Sailer CA, Knaus HG, Gutman GA, Barish ME, LaFerla FM, Chandy KG (2004) Enhanced neuronal excitability in the absence of neurodegeneration induces cerebellar ataxia. JClinInvest 113:582-590.
Soong BW, Paulson HL (2007) Spinocerebellar ataxias: an update. Current opinion in neurology 20:438-446.

Walter JT, Khodakhah K (2006) The linear computational algorithm of cerebellar Purkinje cells. JNeurosci 26:12861-12872.

Walter JT, Khodakhah K (2009) The advantages of linear information processing for cerebellar computation. ProcNatlAcadSciUSA 106:4471-4476.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. NatNeurosci 9:389-397.

Wilson SM, Bhattacharyya B, Rachel RA, Coppola V, Tessarollo L, Householder DB, Fletcher CF, Miller RJ, Copeland NG, Jenkins NA (2002) Synaptic defects in ataxia mice result from a mutation in Usp14, encoding a ubiquitin-specific protease. Nature genetics 32:420-425.

Womack MD, Khodakhah K (2003) Somatic and dendritic small-conductance calcium-activated potassium channels regulate the output of cerebellar purkinje neurons. JNeurosci 23:2600-2607.

Yoon KW, Covey DF, Rothman SM (1993) Multiple mechanisms of picrotoxin block of GABA-induced currents in rat hippocampal neurons. JPhysiol 464:423-439.

Zuberi SM, Eunson LH, Spauschus A, De Silva R, Tolmie J, Wood NW, McWilliam RC, Stephenson JB, Kullmann DM, Hanna MG (1999) A novel mutation in the human voltage-gated potassium channel gene (Kv1.1) associates with episodic ataxia type 1 and sometimes with partial epilepsy. Brain : a journal of neurology 122 (Pt 5):817-825.

FIGURES

FIGURE 1

Purkinje cell and DCN firing defects in *Klh1* knockout mouse *in vivo*.

The firing rate of Purkinje cells (***A***) and DCN neurons (***B***) was measured by extracellular recordings in anaesthetized KO and WT mice *in vivo*. Shown are sample records for each, individual firing rates and coefficients of variation, and mean ± S.E.M. The inset in (***A***) shows the complex spike rate in the same neurons.

*** denotes statistical significance at P<0.0001, ** at P<0.01, * at P<0.05. The inset in panel (***A***) shows the rate of complex spike activity. Throughout the figures, gray denotes WT, purple denotes KO.

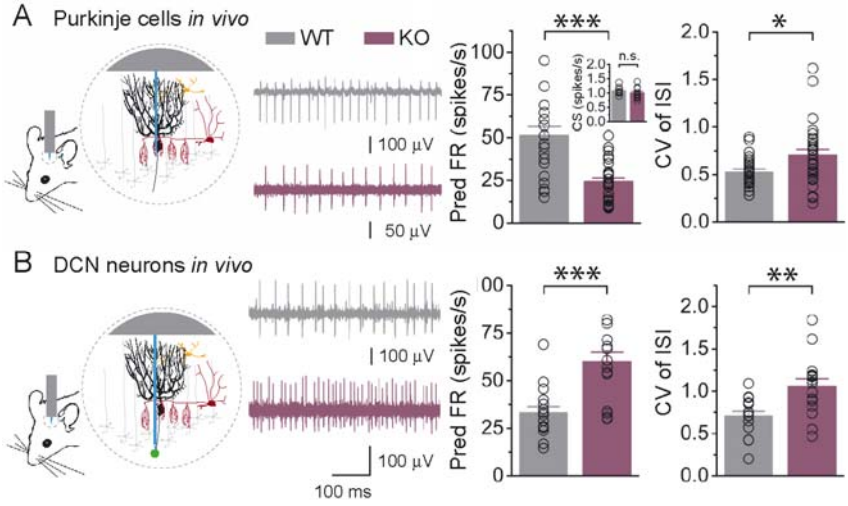

A
Purkinje cells in vivo
WT
KO
100 µV
50 µV
Pred FR (spikes/s)
***
CS (spikes/s)
n.s.
CV of ISI
*
B
DCN neurons in vivo
100 µV
100 µV
100 ms
Pred FR (spikes/s)
***
CV of ISI
**

FIGURE 2

The lower firing rate of knockout Purkinje cells persists in cerebellar slices *in vitro*, and is caused by an increase in their GABAergic inputs.

(***A***) The lower firing rates of KO Purkinje cells recorded extracellularly in cerebellar slice *in vitro* were restored to WT levels after adding blockers of inhibitory synaptic transmission (***B***).

Conditions are schematized on the left. Sample raw data are displayed in the middle column. Scattergrams of different cells (open circles) and compiled mean ± SEM predominant spontaneous or intrinsic firing rates are plotted on the right.

** denotes P<0.001, whereas *n.s.* denotes "not significant".

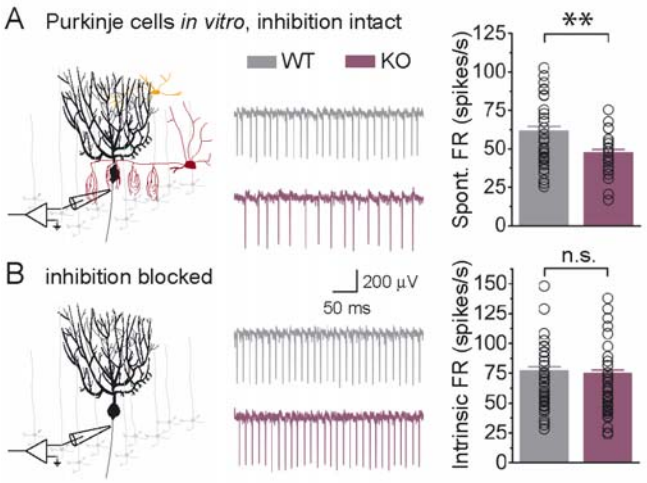

A
Purkinje cells *in vitro*, inhibition intact
WT
KO
**
Spont. FR (spikes/s)
125
100
75
50
25
0
B
inhibition blocked
200 µV
50 ms
n.s.
Intrinsic FR (spikes/s)
150
125
100
75
50
25
0

FIGURE 3

The increased inhibition of knockout Purkinje cells arises from increased frequency of action potential-dependent GABA release.

(***A***) *Top,* Schematic of whole-cell voltage-clamp recording from Purkinje cells and sample raw spontaneous and miniature inhibitory post-synaptic currents in the KO (purple) and WT (gray) Purkinje cells.

(***B***) Purkinje cells show a significant increase in the frequency of sIPSCs in the KO compared to WT but no change in the frequency of mIPSCs.

(***C***) The mean amplitudes of neither sIPCS, nor mIPSCs were significantly different in KO Purkinje cells from those of the WT.

* denotes $P<0.05$, whereas *n.s.* denotes "not significant".

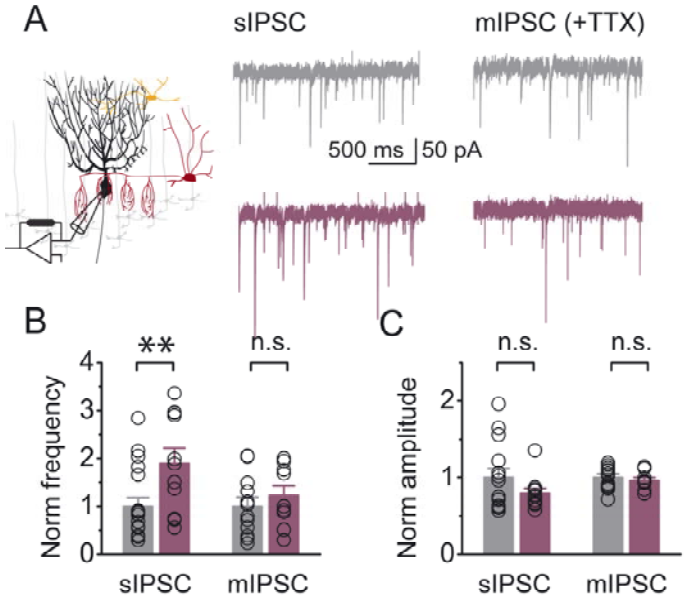

A
sIPSC
mIPSC (+TTX)
500 ms
50 pA
B
**
n.s.
Norm frequency
4
3
2
1
0
sIPSC
mIPSC
C
n.s.
n.s.
Norm amplitude
2
1
0
sIPSC
mIPSC

FIGURE 4

The firing rate of knockout molecular layer interneurons is higher than wild-type cells *in vivo*, while release probabilities at their synapses are comparable.

(***A***) The activity of molecular layer interneurons in the KO mice *in vivo* was significantly higher than that in the WT.

(***B***) Purkinje cell IPSCs were evoked in response to paired electrical stimulation of presynaptic interneurons in voltage-clamped Purkinje cells in cerebellar slices (see schematic). The raw traces are examples of superimposed IPSCs normalized to the peak WT amplitude. There was no difference in the ratios of the second peak to the first, suggesting that the probability of GABA release at the KO and WT synapses were comparable.

*** denotes $P<0.0001$, whereas *n.s.* denotes "not significant".

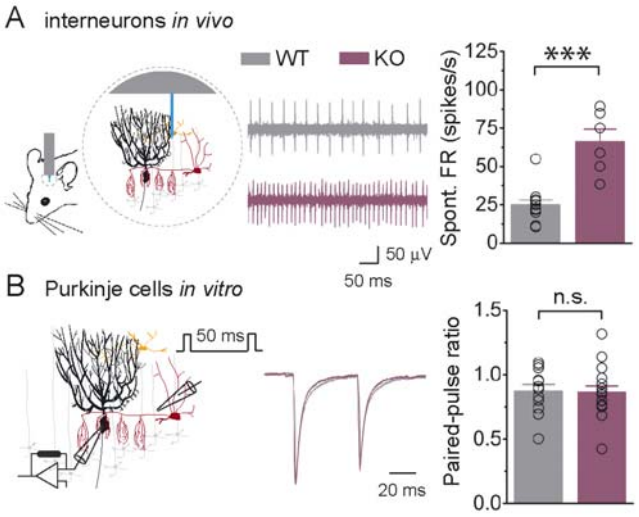

A interneurons *in vivo*
WT
KO
50 µV
50 ms
Spont. FR (spikes/s)
125
100
75
50
25
0
***
B Purkinje cells *in vitro*
50 ms
20 ms
Paired-pulse ratio
1.5
1.0
0.5
0.0
n.s.

FIGURE 5

The knockout Purkinje cells are more excitable than wild-type due to changes in their passive electrical membrane properties.

(***A***) Schematic of sequential voltage-clamp and current-clamp recording and photostimulation paradigm.

(***B***) Sample EPSCs (*left*) and EPSPs (*right*) in response to incremental glutamate photorelease over Purkinje cell dendrites are superimposed.

(***C***) Compiled data across cells are plotted as mean EPSP amplitudes at each photolysis intensity, as a function of mean EPSC amplitudes at the same photolysis intensities.

(***D***) A plot of the normalized ratios of the EPSP peaks of the KO over WT at each intensity.

(***E***) KO Purkinje cells exhibit, on average, a lower dendritic capacitance (C_m) and higher dendritic input resistance (R_{in}) compared to WT.

(***F***) Volume rendering of Purkinje cell dendrites in WT and KO mice.

(***G***) Purkinje cell dendritic volumes in WT and KO mice.

* denotes $P<0.05$, whereas *** denotes $P<0.0001$.

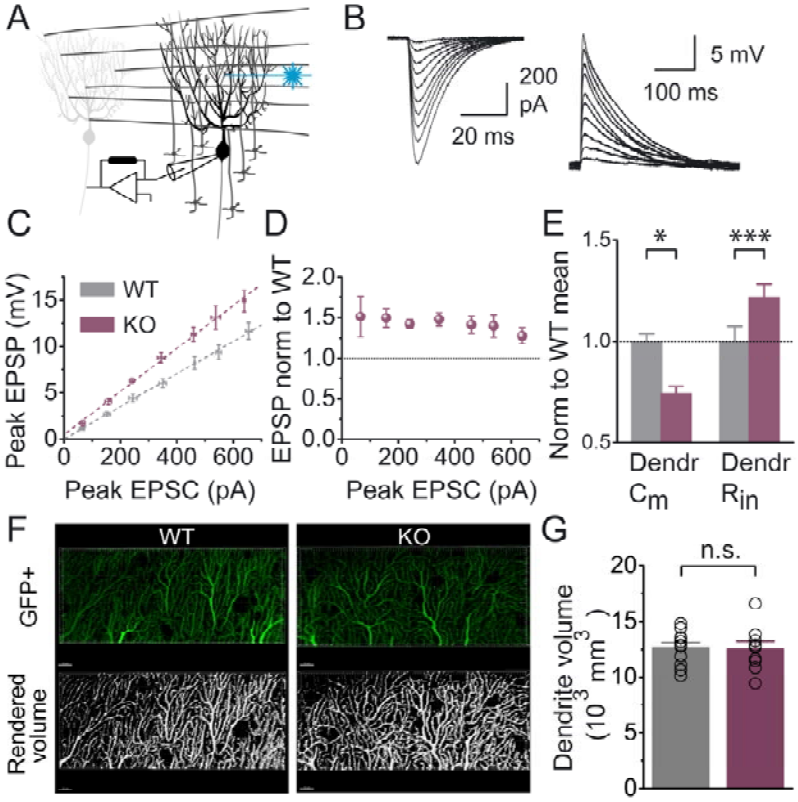

A
B
200
pA
20 ms
5 mV
100 ms
C
D
E
WT
KO
Peak EPSP (mV)
Peak EPSC (pA)
EPSP norm to WT
Peak EPSC (pA)
Norm to WT mean
*
***
Dendr
Cm
Dendr
Rin
F
G
WT
KO
GFP+
Rendered volume
n.s.
Dendrite volume
(10^3 mm^3)

FIGURE 6

The predominant firing rate of DCN neurons of knockout mice is close to wild type in the awake head-restrained preparation.

The firing rate of DCN neurons was measured by extracellular recordings in the awake head-restrained KO and WT mice *in vivo*. Shown are sample records for each, individual firing rates and coefficient of variation, and mean ± S.E.M

* denotes $P<0.05$, whereas *n.s.* denotes "not significant".

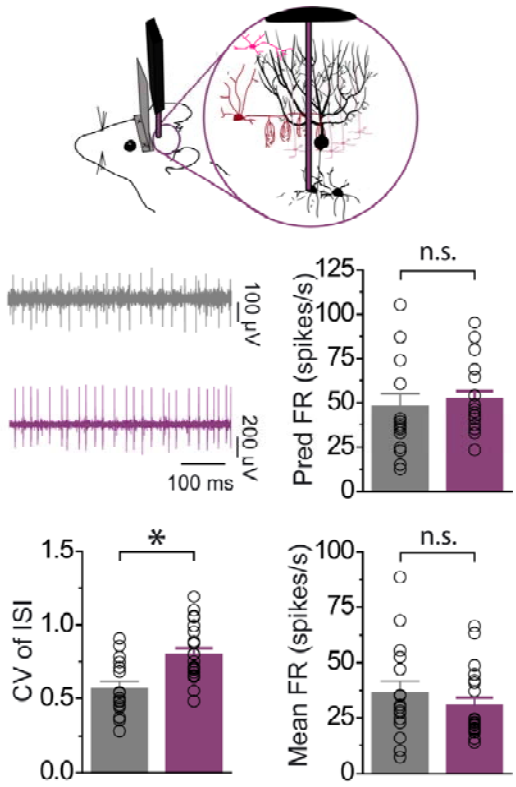

100 μV
200 μV
100 ms
n.s.
Pred FR (spikes/s)
125
100
75
50
25
0
*
CV of ISI
1.5
1.0
0.5
0.0
n.s.
Mean FR (spikes/s)
100
75
50
25
0

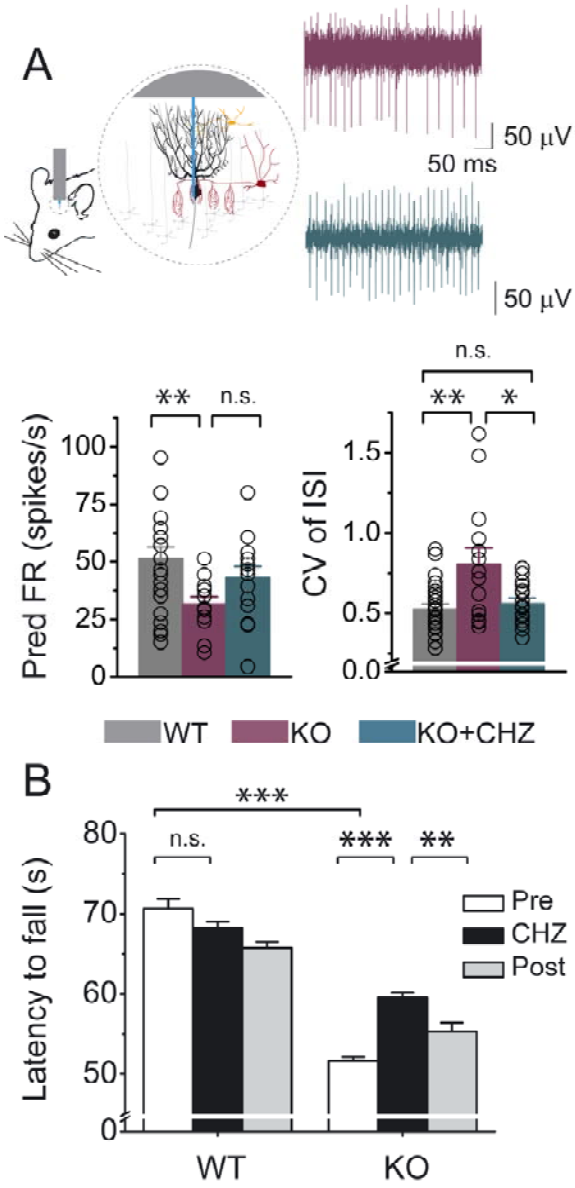
FIGURE 7

Oral administration of chlorzoxazone improves motor performance and firing characteristics of Purkinje cells from KO mice.

(***A***) Mean predominant firing rates and coefficients of variation of Purkinje cells from CHZ-treated KO animals recorded *in vivo*.

(***B***) Performance of KO (n=4) and WT (n=6) animals on an accelerating rotarod and the impact of CHZ oral treatment. Mean latency to fall before (pre, 12 days), during (CHZ, 15 days), and after (post, 10 days) CHZ administration to both groups.

* denotes $P<0.05$, whereas ** denotes $P<0.01$.


A
50 μV
50 ms
50 μV
n.s.
**
**
n.s.
*
Pred FR (spikes/s)
100
75
50
25
0
CV of ISI
1.5
1.0
0.5
0.0
WT
KO
KO+CHZ
B
***
n.s.
***
**
Latency to fall (s)
80
70
60
50
0
Pre
CHZ
Post
WT
KO

CHAPTER V:

ALTERED CEREBELLAR ACTIVITY IN THE ABSENCE OF MORPHOLOGICAL CHANGES IN THE *CAR8* MUTANT MOUSE

Esra Tara, Samrawit Gebre[1], Kamran Khodakhah and Roy Sillitoe

[1] Samrawit Gebre performed the histology and behavioral experiments

ABSTRACT

An overarching feature of inherited cerebellar disease is that mutations causing ataxia often impair the development of the cerebellum or the survival of its neurons as well. The link between genetics, abnormal cerebellar development or neuronal degeneration and the expression of motor symptoms is unclear. In many cases the extent of morphological defects do not correlate with the severity of the motor symptoms, which indicates that to understand the sequence of events that lead to motor dysfunction, the mutations' effect on the proper functioning of cerebellar neurons need to be examined as well. The *waddles* mouse carries a genetic mutation that in humans hinders the development of the cerebellum and gives rise to severe ataxia and mental retardation. We sought to determine whether changes in the behavior of cerebellar neurons could account for the expression of ataxic symptoms. We found cerebellar activity to be markedly altered in the *waddles* mouse, with an erratic output from the cerebellum and burst firing at the level of Purkinje cells. This led us to evaluate the possibility of alleviating the associated motor symptoms by restoring cerebellar output to normalcy.

INTRODUCTION

The majority of inherited cerebellar syndromes involve changes in the morphology of the cerebellum. Almost all autosomal dominant ataxias can be classified as either degenerative or non-degenerative and the clinical picture may be further complicated by the occurrence of congenital malformations. As a result, the exact pathogenic mechanisms contributing to the expression of symptoms is poorly understood. While neurodegeneration is a shared feature of the autosomal dominant spinocerebellar ataxias, abnormalities in the development of the cerebellum commonly occur in autosomal recessive syndromes (Ramaekers et al., 1997; Wassmer et al., 2003). These include some common forms such as Friedreich's ataxia and ataxia telangiectasia and in addition, it is thought that there are numerous less well characterized autosomal recessive syndromes that arise in small, isolated populations or consanguineous families (Ramaekers et al., 1997; Palau and Espinos, 2006; Manto, 2010; Anheim et al., 2012).

Mutations in the gene coding for the carbonic anhydrase-related protein VIII (*CA8* in humans, *car8* in mice) underlie one such disorder. In humans *CA8* mutations belong to the family of inherited cerebellar hypoplasia which share the feature of a decreased cerebellar volume at birth. In general, the extent of hypoplasia is highly variable among affected individuals, with some presenting with almost perfectly formed cerebella and others missing large parts of it (Turkmen et al., 2009; Kaya et al., 2011; Najmabadi et al., 2011). The symptoms accompanying this mutation include borderline to moderate mental retardation as well as severe non-progressive ataxia. In addition, problems with balance cause all affected individuals to learn to walk later in life or not at all, and to commonly fall even from a sitting position (Kaya et al., 2011). Although few

affected families have been reported, it has been noted that within a single family, the extent of morphological abnormality did not correlate with the severity of the ataxia (Kaya et al., 2011).

The *waddles* mouse is a spontaneous mutant discovered in the Jackson Laboratory due its wobbly side-to-side gait and appendicular dystonia (http://www.jax.org/mmr/waddler.html). Genetic analysis had mapped the autosomal recessive *waddles* mutation to exon 19 of the *car8* gene well before its characterization as part of a human syndrome. Both symptoms are present and stable throughout the mouse's lifetime, and seem to replicate the human motor phenotype (Jiao et al., 2005).

The link between a given mutation, the ensuing neuronal degeneration or structural abnormalities and the expression of ataxic symptoms is poorly understood. For example in ataxias associated with neurodegeneration, the onset or severity of symptoms may not always correlate with that of cell death, and in genetic mouse models symptoms may occur in the absence of degeneration (Clark et al., 1997; Dohlinger et al., 2008; Shakkottai et al., 2011). This suggests that the symptoms likely result from cerebellar dysfunction rather than neuronal degeneration. An overarching hypothesis tested in this thesis is that the expression of motor symptoms is ultimately associated with an aberrant output from the cerebellum. Thus, in chapter 2, the cerebellar physiology associated with a non-degenerative inherited cerebellar disorder was studied. Chapter 4 examined the same cerebellar properties associated with symptoms thought to arise from progressive neuronal cell death. Due to the occurrence of symptoms in the absence of cell death, it was concluded that it is aberrant cerebellar physiology rather than loss of neurons that accounted for motor symptoms. Given the marked developmental deficits associated with

the *CA8* mutation in humans, we sought to examine whether changes in the behavior of cerebellar neurons are similarly implicated in the *waddles* mutant's motor symptoms.

METHODS

In vivo electrophysiology: Mice were prepared for chronic recordings under isofluorane anesthesia. A custom made bracket was fixed onto the skull with three bone screws (Plastics One Inc.) and dental cement (M&S Dental Supply). A recording chamber was drilled in the skull above the cerebellum, surrounded with dental cement and covered with surgi-foam (Ethicon) and bone wax (Ethicon).

To record neural activity, the head was immobilized by fixing the head bracket with a screw to the stereotaxic apparatus, the surgi-foam and bone wax were removed and the recording chamber filled with agar. The extracellular activity of single Purkinje cells and DCN neurons were also recorded from mice under isofluorane anesthesia.

Single-unit activity of DCN and Purkinje cells were recorded using a platinum-quartz electrode (2-3 MΩ, Thomas Recording GmbH), which was advanced into the cerebellum until either the Purkinje cell layer or DCN were reached. Purkinje cells were identified by the brief pause in their activity following each complex spike, DCN cells by their location; lack of complex spikes and characteristic firing rate *in vivo* (Hoebeek et al., 2008).

Neural signals were band pass filtered (80 Hz – 20 kHz), amplified (2000×) and digitized (20 kHz) using a data acquisition card (PCI-MIO-16XE) and an in house written software based on LabView (National Instruments, Austin, TX). Waveforms were sorted using Offline Sorter software (Plexon Inc, Dallas, TX USA) using principle component analysis.

Chlorzoxazone administration: During treatment phase, the animals' drinking water was substituted with 15 mM chlorzoxazone (Sigma) in 0.1% hydroxypropyl-β-

cyclodextrin (Tocris) and 10% sucrose, as described in (Alvina and Khodakhah, 2010b). Fresh solution was prepared daily and administered by placing a graduated 15 ml plastic tube inside the animals' cage to facilitate measurement of intake. The animals' weight and water intake were recorded daily.

Perfusion and sectioning: mice were anesthetized with avertin. Once all reflexes were abolished (e.g. lack of blink and corneal reflexes) the blood was flushed through the heart by perfusing with 0.1M phosphate buffered saline (PBS; pH7.2). The tissue was then fixed by perfusing with 4% paraformaldehyde (4% PFA) diluted in PBS. The brains were then postfixed for 24-48 hours in 4% PFA and then cryoprotected in buffered sucrose solutions (15% and 30% diluted in PBS). Serial 40 μm thick coronal and sagittal sections were cut on a cryostat and collected as free-floating sections in PBS.

Immunohistochemistry: immunohistochemistry was carried out as described previously (Sillitoe et al. 2003, 2008b). Briefly, tissue sections were washed thoroughly, blocked with 10% normal goat serum (NGS; Sigma, St. Louis MO) for 1 h at room temperature and then incubated in 0.1 M PBS containing 10% NGS, 0.1% Tween-20 and the primary antibodies for 16–18 h at room temperature. The tissue sections were then washed three times in PBS and incubated in secondary antibodies for 2 h at room temperature. The tissue was rinsed again and immunoreactivity revealed as described below.

Antibodies: Purkinje cells were labeled with rabbit polyclonal anti-Calbindin (Cat# CB-38a; 1:10,000; Swant, Bellinzona, Switzerland), mouse monoclonal anti-Calbindin (Cat# 300; 1:10000; Swant, Bellinzona, Switzerland) and mouse monoclonal anti-CaVIII (Cat. # sc-166626; 1:3000; Santa Cruz, Biotechnology, Santa Cruz, CA),

rabbit polyclonal anti-CaVIII (Cat# sc-67330, Santa Cruz Biotechnology, Santa Cruz, CA). The two different Calbindin and CaVIII antibodies revealed an identical staining pattern and were used interchangeably throughout the study. The primary antibodies were then bound with horseradish peroxidase (HRP) conjugated goat anti-rabbit and goat anti-mouse (diluted 1:200 in PBS; DAKO, Carpinteria, CA) secondary antibodies. Peroxidase binding sites were revealed by incubating in a freshly-prepared 3,3' -diaminobenzidine (DAB; Sigma, St Louis, MO) solution consisting of 1 X 100 mg DAB tablet in 40 ml 1 X PBS and 10 µl 30% H_2O_2. The reaction was stopped and quenched when the correct color intensity was reached. Gross cerebellar morphology was analyzed by staining sagittal cut sections with fluorescent nissl (1: 200; NeuroTrace Cat# N-21480, Molecular Probes, Eugene, OR).

Western Blotting: a standard SDS page western blotting procedure was used to detect Car8 protein in wild type and waddles mutant mice. The details can be found in Sillitoe et al., (2003).

Microscopy and image analysis: photomicrographs of tissue sections were captured using a Leica DFC360 FX camera mounted on a Leica DM5500 microscope, which is equipped with Leica CY3 (model # 11600231), FITC (model # 11513880), and A4 DAPI/UV (model # 11504162) filters. Images of tissue sections were acquired and analyzed using a Leica Application Suite FX software package. All raw data were imported into Adobe Photoshop CS4 and corrected for brightness and contrast only.

RESULTS

Car8 mutation is not associated with morphological defects

Loss of *car8* gene function in the spontaneous mutant *waddles* results in overt ataxia, a phenotype that is largely ascribed to defects in the cerebellum. Cerebellar ataxia is often associated with neuronal degeneration and/or defects in the gross structure and patterning of lobules. To elucidate whether such cellular alterations underlie *car8* ataxia we performed an in depth protein expression and histological study in adult *waddles* mice. The car8 protein is comprised of 291 amino acids and has a predicted molecular weight of ~33kDa. We have found using SDS-page western blotting with monoclonal and polyclonal antibodies that in wild type cerebella the car8 protein migrates at ~36kDa (Figure 1a). The protein is completely absent in whole cerebellum lysates from *waddles* mutants (Figure 1a). In wild type mice, car8 protein is heavily expressed in Purkinje cells (Figure 1b). Immunohistochemical staining of *waddles* mutants demonstrates that car8 protein is indeed absent from Purkinje cells (Figure 1b). Interestingly, the absence of *car8* does not result in gross morphological defects as demonstrated by fluorescent nissl staining of sagittal section cut through the midline of the cerebellum (Figure 1c). Remarkably, immunohistochemical staining with the pan-Purkinje cell marker calbindin revealed that loss of *car8* does not result in Purkinje cell degeneration either (Figure 1d). Together, these data demonstrate that car8 is heavily expressed in cerebellar Purkinje cells, although its loss does not cause gross structural defects or degeneration in the cerebellum.

Altered cerebellar output in the *waddles* mutant

To determine if the *waddles* mutation is accompanied by a change in the output from the cerebellum, we recorded the activity of DCN neurons in awake head-restrained mutants and age-matched wild type controls (Figure 2a). We found the firing pattern of DCN neurons to be highly erratic, as shown by a significant increase in the predominant firing rate of mutants relative to wild types (*waddles*: 71 ± 10 spikes/s, n = 10, N = 2; wild type: 58 ± 4 spikes/s, n = 26, N = 2; $p < 0.05$; Figure 2b), as well as interspike interval coefficient of variation (*waddles*: 1.2 ± 0.14; wild type: 0.71 ± 0.04; $p < 0.001$; Figure 2c). Although there was a small decrease in the mean firing rate as well, this was not significant (*waddles*: 30.7 ± 5.4 spikes/s; wild type: 41 ± 5 spikes/s; $p = 0.3$; Figure 2d). In the absence of a decrease in the mean firing rate, the altered predominant firing rate suggests the presence of periods of high frequency firing as well as low frequency firing or pauses, such that on average the mean firing rate was not significantly altered.

Expression of the *car8* gene has been shown to be restricted to the cerebellum with high expression levels in Purkinje cell soma, axon and dendritic arbor, low levels in the molecular and granule cell layers and virtually no expression elsewhere in the brain (Kato, 1990). We found the expression of the protein product to virtually be limited to Purkinje cells as well, suggesting that the abnormal DCN firing pattern might be driven by an altered output from the cerebellar cortex. Under the same experimental conditions, we recorded the activity of Purkinje cells (Figure 1e) and found their firing pattern to be highly erratic as well, with a similar increase in the predominant firing rate of mutants relative to controls (*waddles*: 69 ± 4 spikes/s, n = 14, N=2; wild type: 50.4 ± 7, n = 18; N = 2; $p < 0.04$; Figure 2f) and an even larger increase in the CV of ISIs (*waddles*: 1.4 ±

0.15; wild type: 0.6 ± 0.05; $p < 0.001$; Figure 2g) without an accompanying change in the mean firing rate (*waddles*: 43 ± 4.19 spikes/s; wild type: 37 ± 5 spikes/s; $p = 0.7$; Figure 2h).

Increased regularity of Purkinje cell firing accompanied by improved motor function

If erratic Purkinje cell activity underlies the motor symptoms observed in the *waddles* mouse, we reasoned that pharmacologically restoring the regularity of Purkinje cell firing should alleviate this symptom. Based on the findings in Chapter 2 and 4, as well as prior studies in the literature indicating the efficacy with which K_{Ca} channel activators decrease the excitability of Purkinje cells in ataxic mutant mice (Walter et al., 2006; Alvina and Khodakhah, 2010a; Shakkottai et al., 2011), we examined whether a similar approach could be used to alleviate the *waddles* mutant's motor symptoms. We thus recorded the firing of Purkinje cells *in vivo* in mice whose drinking water had been supplemented with the K_{Ca} channel activator chlorzoxazone (CHZ) (Cao et al., 2001). Following a 7-day course of treatment with CHZ, we found the firing pattern of Purkinje cells in anesthetized *waddles* mice to be significantly less erratic (Figure 2a). This was characterized by a predominant firing rate virtually indistinguishable from that of wild type mice (*waddles*: 72 ± 6 spikes/s, n = 15, N = 2; *waddles* + CHZ: 53 ± 10 spikes/s, n = 12, N = 2; wild type: 56 ± 5 spikes/s, n = 22, N = 2; wild type + CHZ: 48 ± 8 spikes/s, n = 10, N = 2; $p = 0.7$ compared to treated mutants; Figure 2b) and a similar reduction in the CV of ISIs (*waddles*: 1.2 ± 0.16; *waddles* + CHZ: 0.56 ± 0.04; wild type: 0.6 ± 0.05; wild type + CHZ: 0.58 ± 0.03; $p = 0.7$ compared to treated mutants; Figure 2c), while the mean firing rate remained unchanged (*waddles*: 42 ± 6 spikes/s; *waddles* +

CHZ: 40.97 ± 7 spikes/s; wild type: 41 ± 4 spikes/s; wild type + CHZ: 32.75 ± 3.7 spikes/s; $p = 0.3$ compared to treated mutants; Figure 2d).

To determine whether the administration of CHZ translated to an improvement in the mutant's motor symptoms, we used an accelerating rotarod paradigm to assess motor function before, during and after oral administration of CHZ to mutants and wild type controls. In agreement with previous findings (Jiao et al., 2005), we found the *waddles* mutation to be accompanied by significant motor dysfunction apparent in the mutants decreased latency to fall from the accelerating rotarod (mean latency to fall: *waddles* untreated = 26 ± 2 s, N=5; wild type untreated = 51 ± 5 s; $p < 0.001$, N=5). In response to treatment with CHZ, the performance of the mutants markedly improved (mean latency to fall: *waddles* + CHZ = 36 ± 2 s; N = 5; $p < 0.001$ compared to untreated mutants, Figure 3d) without a change in the performance of wild types (mean latency to fall: wild type + CHZ = 49 ± 3 s, N=5; $p = 0.1$ compared to untreated wild types, Figure 3d).

The improvement in the motor phenotype upon administration of CHZ was associated with a decreased predominant firing rate of mutant DCN neurons (wild type: 34 ± 4 spikes/s; n = 16; N = 2; untreated *waddles*: 71 ± 7 spikes/s; n = 11, N = 2; *waddles* + CHZ: 45 ± 6 spikes/s n = 8, N = 1; $p < 0.05$ compared to untreated mutants Figure 4a) and CV of ISIs (wild type: 0.7 ± 0.06; *waddles*: 1.1 ± 0.13; *waddles* + CHZ: 0.72 ± 0.05; $p < 0.05$ compared to untreated mutants; Figure 4b), further strengthening the correlation between the aberrant cerebellar output pattern and the expression of symptoms.

DISCUSSION

A wide range of genetic mutations give rise to ataxia and most seem to damage the cerebellum on multiple levels. Thus many forms of inherited ataxia are accompanied by neuronal degeneration or developmental abnormalities (Paulson, 2009; Ikeda et al., 2012; Seidel et al., 2012). The mechanism by which such changes ultimately give rise to ataxia is unclear. Moreover, in a subset of these, mouse models that replicate the genetic defects have the same motor symptoms in the absence of cell death (Clark et al., 1997; Shakkottai et al., 2011). Similarly, in inherited forms of cerebellar hypoplasia, the extent of morphological abnormalities does not necessarily correlate with the severity of the symptoms (Dudding et al., 2004; Yapici and Eraksoy, 2005; Tsao et al., 2006; Kaya et al., 2011; Zerem et al., 2012). In order to understand the sequence of events that lead to motor dysfunction, the outcome of genetic defects on the functioning of neurons need to be examined as well. Here we show that a mutation in the *car8* gene does not induce the degeneration of cerebellar neurons in the *waddles* mouse. On the other hand, we found the behavior of cerebellar neurons to be markedly altered with a highly erratic output from the cerebellum and burst firing at the level of Purkinje cells. Further strengthening the correlation between aberrant Purkinje cell activity and the expression of symptoms, we found the oral administration of a K_{Ca} channel activator to restore Purkinje cell firing to normalcy and alleviate the mutant's motor symptoms.

Absence of Purkinje cell degeneration in *waddles* mutants

The *car8* protein product was originally thought to belong to the larger family of carbonic anhydrases due to structural similarities (Kato, 1990) but subsequent studies indicate it lacks the enzymatic carbonic anhydrase activity (Sjoblom et al., 1996).

Car8 is predominantly expressed in cerebellar Purkinje cells (Kato, 1990) and it is now known that it binds to the regulatory subunit of inositol triphosphate receptor-1 (IP_3R1) an intracellular IP_3-gated calcium channel abundantly expressed at this location (Furuichi et al., 1993; Hirota et al., 2003). The binding of car8 protein lowers the affinity of IP_3 for its receptor (Hirota et al., 2003), presumably resulting in a decreased release of calcium from intracellular stores. Although the functional implications of this interaction are unknown, it is thought that the inhibitory effect car8 exerts on IP_3 receptors may account for the fact that much higher concentrations of IP_3 are required for IP_3-induced calcium release in Purkinje cells relative to other neurons (Hirota et al., 2003).

The *CA8* syndrome adds to the growing list of disorders implicating the proper functioning of IP_3 receptors specifically and calcium signaling generally in the pathomechanism of inherited ataxias. Thus, mice lacking IP_3Rs are severely ataxic (Matsumoto et al., 1996) and a spontaneous mutation in the gene encoding the type 1 IP_3R is also accompanied by severe ataxia in the opisthotonos mouse (Street et al., 1997). Intriguingly, mutations in or total deletion of IP_3Rs in humans cause spinocerebellar ataxia 15 which is characterized by ataxia and cerebellar hypoplasia (Dudding et al., 2004; van de Leemput et al., 2007; Hara et al., 2008; Marelli et al., 2011; Zerem et al., 2012). Moreover, the protein products of the mutated genes in spinocerebellar ataxia 2 and 3 have recently been shown to bind to the carboxy-terminal region of the type 1 IP_3R (Chen et al., 2008; Liu et al., 2009; Kasumu and Bezprozvanny, 2010). The functional consequence of this interaction is an increased sensitivity of IP_3R1 to IP_3 binding which results in an increased release of calcium from intracellular calcium stores (Chen et al., 2008; Liu et al., 2009; Kasumu and Bezprozvanny, 2010). Based on these findings, it has

been hypothesized that the characteristic neuronal degeneration in spinocerebellar ataxia results from a toxic effect of excessive intracellular calcium levels (Bezprozvanny, 2009). The binding of car8 protein lowers the affinity of IP_3 for its receptor (Hirota et al., 2003), presumably resulting in a decreased release of calcium from intracellular stores. We found the car8 protein to be entirely absent in *waddles* Purkinje cells, which would be predicted to result in an increased sensitization of the IP_3R to IP_3 as well. Surprisingly, we did not find the loss of car8 protein from Purkinje cells to associate with neuronal loss. The apparent dissonance between our results and the predictions that arise from earlier studies suggests that in the *waddles* mouse either compensatory mechanisms prevent calcium homeostasis from being altered or that in this case altered calcium homeostasis does not produce toxic effects on cerebellar neurons. Calcium imaging studies would help determine whether the loss of car8 protein indeed results in the sensitization of IP_3-induced release of calcium. Given the involvement of intracellular calcium in a wide range of cellular processes, it is also possibly that car8 loss of function does not exert its role by altering IP_3R function but through cellular processes that are independent of its immediate effects on the receptor.

Altered cerebellar activity in *waddles* mutants

We found mutant Purkinje cells to fire erratically and the increased activation of K_{Ca} channels to alleviate the motor deficits of the *waddles* mouse. There are numerous ways in which the firing of Purkinje cells can be altered in this manner. IP_3 receptors play a crucial role in the regulation of intracellular calcium signaling, which is in turn

involved in a wide range of cellular processes including synaptic plasticity at the parallel fiber-Purkinje cell synapse (Khodakhah and Armstrong, 1996; Finch et al., 2011). This forms the basis of a second hypothesis proposed to explain the frequency with which IP_3R-mediated calcium signaling is altered in ataxias. Thus, it has been argued that most ataxia mutations can be linked to synaptic plasticity deficits by way of their deleterious effects on Purkinje cell calcium signaling (Schorge et al., 2010). In support of this hypothesis, the architecture of parallel fiber-Purkinje cell synapse is altered in many ataxic mice. For example the targeting of parallel fibers onto Purkinje cell spines is impaired in mice deficient in the GluRδ2 subunit of glutamate receptors (Hirano, 2012). These mice are also ataxic (Yoshida et al., 2004). In fact, a recent study found the ultrastructural morphology of the cerebellum to be disrupted in *waddles* as well, such that mutant Purkinje cells had significantly more free spines that in wild types form synapses with parallel fibers (Hirasawa et al., 2007). While this may alter plasticity at the parallel fiber-Purkinje cell synapse, current evidence argues against a major role for synaptic plasticity deficits in the pathogenic mechanism underlying ataxia. For example, when key proteins involved in synaptic plasticity at the parallel fiber-Purkinje cell synapse are knocked out in mice, long term depression is indeed impaired yet motor coordination remains intact (Miall and Silburn, 1997; De Zeeuw et al., 1998; Feil et al., 2003; Hansel et al., 2006). While among the multifarious genetic mutations that give rise to cerebellar ataxia the probability that some will also impair synaptic plasticity is high, to what extent this would impair motor coordination remains to be determined.

An alternative hypothesis is that irregular Purkinje cell firing causes ataxia by impairing the ability of Purkinje cells to reliably encode synaptic inputs as changes in the

timing of spikes in their output (Hoebeek et al., 2005; Walter et al., 2006). This is supported by the finding that in *cacna1a* mutant mice, a diminished P/Q-type calcium current density decreases the regularity of tonic pacemaking in Purkinje cells and K_{Ca} channel activators, which restore the precision of the intrinsic Purkinje cell activity *in vitro*, alleviate the ataxic symptoms *in vivo* (Walter et al., 2006; Alvina and Khodakhah, 2010b). The finding that chlorzoxazone, a drug that increases the open probability of K_{Ca} channels (Cao et al., 2001), similarly alleviates the motor symptoms in *waddles* mice supports this hypothesis. Moreover that chlorzoxazone regularizes the activity of Purkinje cells *in vivo* extends the findings of previous studies which examined its action *in vitro* (Alvina and Khodakhah, 2010b).

Although our data does not reveal the reason behind the altered Purkinje cell activity, the fact that car8 expression is virtually limited to Purkinje cells implicates their dysfunction in the pathomechanism. Defects in the targeting of parallel fibers to Purkinje cell spines might in fact play a role since erratic Purkinje cell firing is also observed in the GluRδ2 deficient mice (Yoshida et al., 2004). In chapter 4 it was found that in a mouse model of spinocerebellar ataxia 8, a compensatory change in the activity of molecular layer interneurons induces erratic Purkinje cell firing, even though the deletion of the gene in the mouse model is restricted to Purkinje cells. Thus, compensatory changes in upstream neurons providing input to Purkinje cells need to be examined as well.

Cerebellar dysfunction as an instigator of motor symptoms

Our results corroborate findings from a previous study in which light microscopic examination of the *waddles* cerebellum had failed to identify gross morphological defects

(Jiao et al., 2005). Although in humans mutations in *CA8* result in changes in the gross morphology of the cerebellum, it is possible that this is another victim rather than the culprit in the expression of cerebellar motor symptoms. Seminal experiments described in the introduction demonstrated the symptoms that result from gross morphological damage to the cerebellum gradually improve and in many cases disappear over time (Flourens, 1824; Dalton, 1861; Luciani, 1891). Similarly, the symptoms of cerebellar lesions in humans have been noted to gradually improve (Holmes, 1917). This indicates that the cerebellum is a highly plastic structure that is able to compensate for its partial damage. If this is the case, when a portion of the cerebellum is missing at birth, one would expect the remaining cerebellar tissue to compensate and symptoms to disappear over time. In fact this is exactly what has been reported in the literature for many of the rarely occurring cases of sporadic congenital cerebellar hypoplasia (Batten, 1905; Jervis, 1950; Rivier and Echenne, 1992; al Shahwan et al., 1995; Yapici and Eraksoy, 2005; Tsao et al., 2006).

More evidence corroborating this comes from the existence of numerous cases in the literature describing marked cerebellar underdevelopment found upon autopsy in individuals who showed no motor deficits in adulthood (Fusari, 1892; Freeman, 1929). The effects of the mutation at the molecular level remain to be determined. There exists evidence that ultrastructural developmental defects instigate the aberrant behavior of Purkinje cells recorded in *waddles* mice (Hirasawa et al., 2007). Alternatively, the mutation might alter the intrinsic properties of cerebellar neurons. Either way, any change in the wiring of the cerebellum or the excitability of its neurons must translate into a change in the behavior of its output neurons to cause ataxia. Future studies evaluating the

impact of abnormal development of the cerebellum on the physiology of its neurons would be of interest. For example, the *reeler* mouse carries a mutation in the very low-density lipoprotein, which is a component of the reelin pathway (D'Arcangelo et al., 1995). The very low-density lipoprotein gene is mutated in the relatively better characterized CAMRQ-1 syndrome as well (Boycott et al., 2005). In *reeler* mutants, the distinct ataxic gait ("reeling" gait) is associated with several developmental defects including profound hypoplasia of the cerebellum, with absence of normal cerebellar folia (Howell et al., 1997). Thus, these mice would likely be excellent candidates to further investigate the contribution of abnormal physiology to the cerebellar symptoms shared by the syndromes of this family.

An emerging hypothesis with regard to spinocerebellar ataxias is that similar changes in the physiology of cerebellar neurons underlie both the early motor symptoms as well the subsequent cell death associated with these syndromes (Bezprozvanny, 2009; Kasumu and Bezprozvanny, 2010). A similar mechanism might be at play with regard to the *car8* mutation. That *car8* plays an important role in the proper development of the cerebellum is highlighted by the finding that its mutation causes severe morphological abnormalities in humans (Kaya et al., 2011). While in the *waddles* mouse no gross morphological defects were observed under light microscope (Jiao et al., 2005), subsequent electron microscopy studies indicate that synaptogenesis is markedly impaired (Hirasawa et al., 2007). Thus, it would seem that car8 is crucial both for the proper development of the cerebellum as well as the maintenance of normal neuronal function. Based on our results and those of previous studies implicating abnormal

Purkinje cell firing in the pathogenesis of ataxia (Walter et al., 2006), we would speculate that it is the latter effect that ultimately impairs motor function in the *waddles* mouse.

REFERENCES

al Shahwan SA, Bruyn GW, al Deeb SM (1995) Non-progressive familial congenital cerebellar hypoplasia. Journal of the neurological sciences 128:71-77.

Alvina K, Khodakhah K (2010a) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Alvina K, Khodakhah K (2010b) The therapeutic mode of action of 4-aminopyridine in cerebellar ataxia. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7258-7268.

Anheim M, Tranchant C, Koenig M (2012) The autosomal recessive cerebellar ataxias. The New England journal of medicine 366:636-646.

Batten FE (1905) Ataxia in childhood. Brain : a journal of neurology 28:484.
Bezprozvanny I (2009) Calcium signaling and neurodegenerative diseases. Trends in molecular medicine 15:89-100.

Boycott KM, Flavelle S, Bureau A, Glass HC, Fujiwara TM, Wirrell E, Davey K, Chudley AE, Scott JN, McLeod DR, Parboosingh JS (2005) Homozygous deletion of the very low density lipoprotein receptor gene causes autosomal recessive cerebellar hypoplasia with cerebral gyral simplification. American journal of human genetics 77:477-483.

Cao Y, Dreixler JC, Roizen JD, Roberts MT, Houamed KM (2001) Modulation of recombinant small-conductance Ca(2+)-activated K(+) channels by the muscle relaxant chlorzoxazone and structurally related compounds. JPharmacolExpTher 296:683-689.

Chen X, Tang TS, Tu H, Nelson O, Pook M, Hammer R, Nukina N, Bezprozvanny I (2008) Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:12713-12724.

Clark HB, Burright EN, Yunis WS, Larson S, Wilcox C, Hartman B, Matilla A, Zoghbi HY, Orr HT (1997) Purkinje cell expression of a mutant allele of SCA1 in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:7385-7395.

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374:719-723.

Dalton JC (1861) On the cerebellum, as the center of the co-ordination of voluntary movement. Am J Med Sci 61:83-88.

De Zeeuw CI, Hansel C, Bian F, Koekkoek SK, van Alphen AM, Linden DJ, Oberdick J (1998) Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 20:495-508.

Dohlinger S, Hauser TK, Borkert J, Luft AR, Schulz JB (2008) Magnetic resonance imaging in spinocerebellar ataxias. Cerebellum 7:204-214.

Dudding TE, Friend K, Schofield PW, Lee S, Wilkinson IA, Richards RI (2004) Autosomal dominant congenital non-progressive ataxia overlaps with the SCA15 locus. Neurology 63:2288-2292.

Feil R, Hartmann J, Luo C, Wolfsgruber W, Schilling K, Feil S, Barski JJ, Meyer M, Konnerth A, De Zeeuw CI, Hofmann F (2003) Impairment of LTD and cerebellar learning by Purkinje cell-specific ablation of cGMP-dependent protein kinase I. The Journal of cell biology 163:295-302.

Finch EA, Tanaka K, Augustine GJ (2011) Calcium as a Trigger for Cerebellar Long-Term Synaptic Depression. Cerebellum.

Flourens MJP (1824) Researches expérimentales sur le propriétés et fonctions du système nerveux dans les animaux vertébrés. Paris: Cervot.

Freeman W (1929) Cerebellar agenesis: preliminary report of an extreme case. Arch Neurol & Psychiat 22:858-859.

Furuichi T, Simon-Chazottes D, Fujino I, Yamada N, Hasegawa M, Miyawaki A, Yoshikawa S, Guenet JL, Mikoshiba K (1993) Widespread expression of inositol 1,4,5-trisphosphate receptor type 1 gene (Insp3r1) in the mouse central nervous system. Receptors & channels 1:11-24.

Fusari R (1892) Caso di mancaza quasi totale del cervelletto. Atti Accad Sc 2:177-192.
Hansel C, de Jeu M, Belmeguenai A, Houtman SH, Buitendijk GH, Andreev D, De Zeeuw CI, Elgersma Y (2006) alphaCaMKII Is essential for cerebellar LTD and motor learning. Neuron 51:835-843.

Hara K, Shiga A, Nozaki H, Mitsui J, Takahashi Y, Ishiguro H, Yomono H, Kurisaki H, Goto J, Ikeuchi T, Tsuji S, Nishizawa M, Onodera O (2008) Total deletion and a missense mutation of ITPR1 in Japanese SCA15 families. Neurology 71:547-551.

Hirano T (2012) Glutamate-receptor-like molecule GluRdelta2 involved in synapse formation at parallel fiber-Purkinje neuron synapses. Cerebellum 11:71-77.

Hirasawa M, Xu X, Trask RB, Maddatu TP, Johnson BA, Naggert JK, Nishina PM, Ikeda A (2007) Carbonic anhydrase related protein 8 mutation results in aberrant synaptic morphology and excitatory synaptic function in the cerebellum. Molecular and cellular neurosciences 35:161-170.

Hirota J, Ando H, Hamada K, Mikoshiba K (2003) Carbonic anhydrase-related protein is a novel binding protein for inositol 1,4,5-trisphosphate receptor type 1. The Biochemical journal 372:435-441.

Hoebeek FE, Khosrovani S, Witter L, De Zeeuw CI (2008) Purkinje cell input to cerebellar nuclei in tottering: ultrastructure and physiology. Cerebellum 7:547-558.
Holmes G (1917) The symptoms of acute cerebellar injuries due to gunshot injuries. Brain : a journal of neurology 40:461-535.

Howell BW, Hawkes R, Soriano P, Cooper JA (1997) Neuronal position in the developing brain is regulated by mouse disabled-1. Nature 389:733-737.

Ikeda Y, Ranum LP, Day JW (2012) Clinical and genetic features of spinocerebellar ataxia type 8. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:493-505.

Jervis GA (1950) Early familial cerebellar degeneration (report of three cases in one family). J Nerv & Ment Dis 111:398-407.

Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, Gu W (2005) Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. Genetics 171:1239-1246.

Kasumu A, Bezprozvanny I (2010) Deranged Calcium Signaling in Purkinje Cells and Pathogenesis in Spinocerebellar Ataxia 2 (SCA2) and Other Ataxias. Cerebellum.
Kato K (1990) Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. FEBS letters 271:137-140.

Kaya N, Aldhalaan H, Al-Younes B, Colak D, Shuaib T, Al-Mohaileb F, Al-Sugair A, Nester M, Al-Yamani S, Al-Bakheet A, Al-Hashmi N, Al-Sayed M, Meyer B, Jungbluth H, Al-Owain M (2011) Phenotypical spectrum of cerebellar ataxia associated with a novel mutation in the CA8 gene, encoding carbonic anhydrase (CA) VIII. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 156B:826-834.

Khodakhah K, Armstrong C (1996) Induction of LTD by photolytic release of inositol trisphosphate in Purkinje neurones. BiophysJ 70:A196.

Liu J, Tang TS, Tu H, Nelson O, Herndon E, Huynh DP, Pulst SM, Bezprozvanny I (2009) Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type

2. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:9148-9162.

Luciani L (1891) Il cervelletto: nuovi studi di fisiologia normale e patologica. Firenze: Le Monnier.

Manto MU (2010) Cerebellar Disorders. New York: Cambridge University Press.

Marelli C et al. (2011) SCA15 due to large ITPR1 deletions in a cohort of 333 white families with dominant ataxia. Archives of neurology 68:637-643.

Matsumoto M, Nakagawa T, Inoue T, Nagata E, Tanaka K, Takano H, Minowa O, Kuno J, Sakakibara S, Yamada M, Yoneshima H, Miyawaki A, Fukuuchi Y, Furuichi T, Okano H, Mikoshiba K, Noda T (1996) Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5- trisphosphate receptor. Nature 379:168-171.

Miall RC, Silburn P (1997) A study of motor performance and motor learning in episodic ataxia. Neuroreport 8:2159-2164.

Najmabadi H et al. (2011) Deep sequencing reveals 50 novel genes for recessive cognitive disorders. Nature 478:57-63.

Palau F, Espinos C (2006) Autosomal recessive cerebellar ataxias. Orphanet journal of rare diseases 1:47.

Paulson HL (2009) The spinocerebellar ataxias. Journal of neuro-ophthalmology : the official journal of the North American Neuro-Ophthalmology Society 29:227-237.

Ramaekers VT, Heimann G, Reul J, Thron A, Jaeken J (1997) Genetic disorders and cerebellar structural abnormalities in childhood. Brain : a journal of neurology 120 (Pt 10):1739-1751.

Rivier F, Echenne B (1992) Dominantly inherited hypoplasia of the vermis. Neuropediatrics 23:206-208.

Schorge S, van de Leemput J, Singleton A, Houlden H, Hardy J (2010) Human ataxias: a genetic dissection of inositol triphosphate receptor (ITPR1)-dependent signaling. Trends in neurosciences 33:211-219.

Seidel K, Siswanto S, Brunt ER, den Dunnen W, Korf HW, Rub U (2012) Brain pathology of spinocerebellar ataxias. Acta neuropathologica 124:1-21.

Shakkottai VG, do Carmo Costa M, Dell'Orco JM, Sankaranarayanan A, Wulff H, Paulson HL (2011) Early changes in cerebellar physiology accompany motor dysfunction in the polyglutamine disease spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:13002-13014.

Sjoblom B, Elleby B, Wallgren K, Jonsson BH, Lindskog S (1996) Two point mutations convert a catalytically inactive carbonic anhydrase-related protein (CARP) to an active enzyme. FEBS letters 398:322-325.

Street VA, Bosma MM, Demas VP, Regan MR, Lin DD, Robinson LC, Agnew WS, Tempel BL (1997) The type 1 inositol 1,4,5-trisphosphate receptor gene is altered in the opisthotonos mouse. JNeurosci 17:635-645.

Tsao JW, Neal J, Apse K, Stephan MJ, Dobyns WB, Hill RS, Walsh CA, Sheen VL (2006) Cerebellar ataxia with progressive improvement. Archives of neurology 63:594-597.

Turkmen S, Guo G, Garshasbi M, Hoffmann K, Alshalah AJ, Mischung C, Kuss A, Humphrey N, Mundlos S, Robinson PN (2009) CA8 mutations cause a novel syndrome characterized by ataxia and mild mental retardation with predisposition to quadrupedal gait. PLoS genetics 5:e1000487.

van de Leemput J et al. (2007) Deletion at ITPR1 underlies ataxia in mice and spinocerebellar ataxia 15 in humans. PLoS genetics 3:e108.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. Nature neuroscience 9:389-397.

Wassmer E, Davies P, Whitehouse WP, Green SH (2003) Clinical spectrum associated with cerebellar hypoplasia. Pediatric neurology 28:347-351.

Yapici Z, Eraksoy M (2005) Non-progressive congenital ataxia with cerebellar hypoplasia in three families. Acta Paediatr 94:248-253.

Yoshida T, Katoh A, Ohtsuki G, Mishina M, Hirano T (2004) Oscillating Purkinje neuron activity causing involuntary eye movement in a mutant mouse deficient in the glutamate receptor delta2 subunit. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2440-2448.

Zerem A, Hacohen Y, Ben-Sira L, Lev D, Malinger G, Lerman-Sagie T (2012) Dominantly Inherited Nonprogressive Cerebellar Hypoplasia Identified in Utero. Journal of child neurology.

FIGURES

FIGURE 1

Loss of *car8* is not associated with Purkinje cell degeneration.

(a) SDS-page Western blot of wild type and *waddles* cerebellar lysate using car8 antibody. In the wild type cerebella the car8 protein migrates at ~36kDa. The protein is completely absent in whole cerebellum lysates from *waddles* mutants.

(b) Sagittal section through the adult cerebellum immuno-stained with car8 antibody. Left panel: in wild type mice, car8 protein is heavily expressed in Purkinje cells. Right panel: car8 protein is absent from Purkinje cells in the *waddles* mutants.

(c) Fluorescent nissl staining of sagittal section cut through the midline of the cerebellum. No gross morphological changes in *waddles* mutants (right panel) compared to wild type (left panel).

(d) Coronal cerebellar section immuno-stained for calbindin. No loss of Purkinje cells in the *waddles* mutant (right panel).

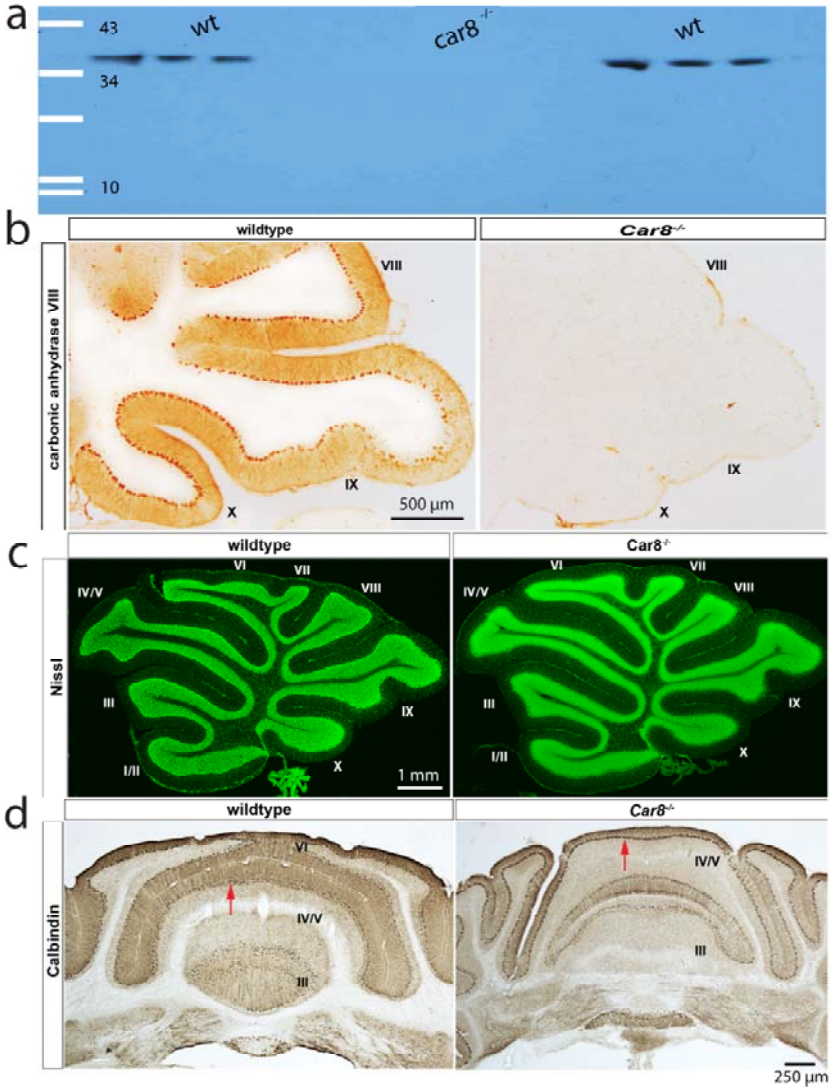

a
43
34
10
wt
Car8 -/-
wt
b
wildtype
Car8-/-
carbonic anhydrase VIII
VIII
IX
X
500 µm
c
wildtype
Car8-/-
Nissl
VI
VII
VIII
IV/V
III
IX
I/II
X
1 mm
d
wildtype
Car8-/-
Calbindin
VI
IV/V
III
250 µm

FIGURE 2

The output of the cerebellum is altered by erratic Purkinje and DCN neuron firing in awake *waddles* mice.

(a) Typical behavior of DCN neurons recorded in wild type and *waddles* mice. illustrating the erratic activity associated with the *waddles* mutation. Individual and average ± S.E.M. values for **(b)** the predominant firing rate, **(c)** coefficient of variation and **(d)** average firing rate of DCN neurons of waddles and wild type mice.

(e) Typical behavior of Purkinje cells recorded in wild type and *waddles* mice Individual and average ± S.E.M. values of **(f)** the predominant firing rate, **(g)** coefficient of variation and **(h)** average firing rate of Purkinje neurons in *waddles* and wild type mice.

** denotes statistical significance at $P<0.001$, * at $P<0.05$, n.s. denotes "not significant". Throughout the figures, gray denotes WT, blue denotes mutant.

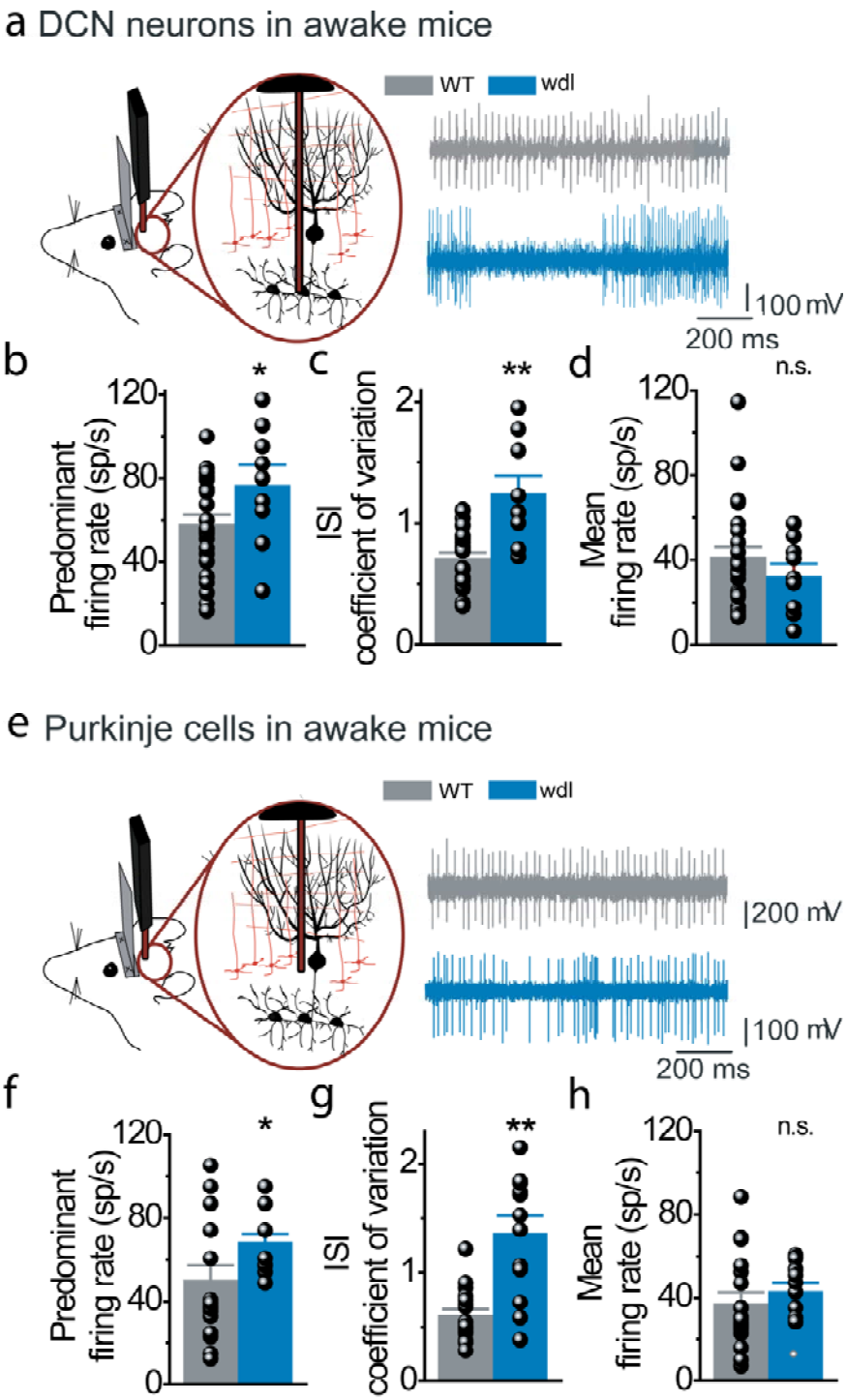

a DCN neurons in awake mice
WT
wdl
100 mV
200 ms
b
Predominant firing rate (sp/s)
120
80
40
0
*
c
ISI coefficient of variation
2
1
0
**
d
Mean firing rate (sp/s)
120
80
40
0
n.s.
e Purkinje cells in awake mice
WT
wdl
200 mV
100 mV
200 ms
f
Predominant firing rate (sp/s)
120
80
40
0
*
g
ISI coefficient of variation
2
1
0
**
h
Mean firing rate (sp/s)
120
80
40
0
n.s.

FIGURE 3

Oral administration of chlorzoxazone improves motor performance and firing characteristics of Purkinje cells from *waddles* mice.

(a) Typical behavior of Purkinje neurons recorded in anesthetized wild type and *waddles* mice following treatment with CHZ. Individual and average ± S.E.M. values for **(b)** the predominant firing rate, **(c)** coefficient of variation and of Purkinje neurons of treated and untreated wild type and *waddles* mice. **(c)** Mean latency to fall of *waddles* and wild type mice before (14 days) and during (10 days) CHZ administration

** denotes statistical significance at $p < 0.001$, n.s. denotes "not significant" $p > 0.05$, gray denotes untreated WT, light gray denotes WT treated with CHZ, blue denotes mutant, light blue denotes mutant treated with CHZ.

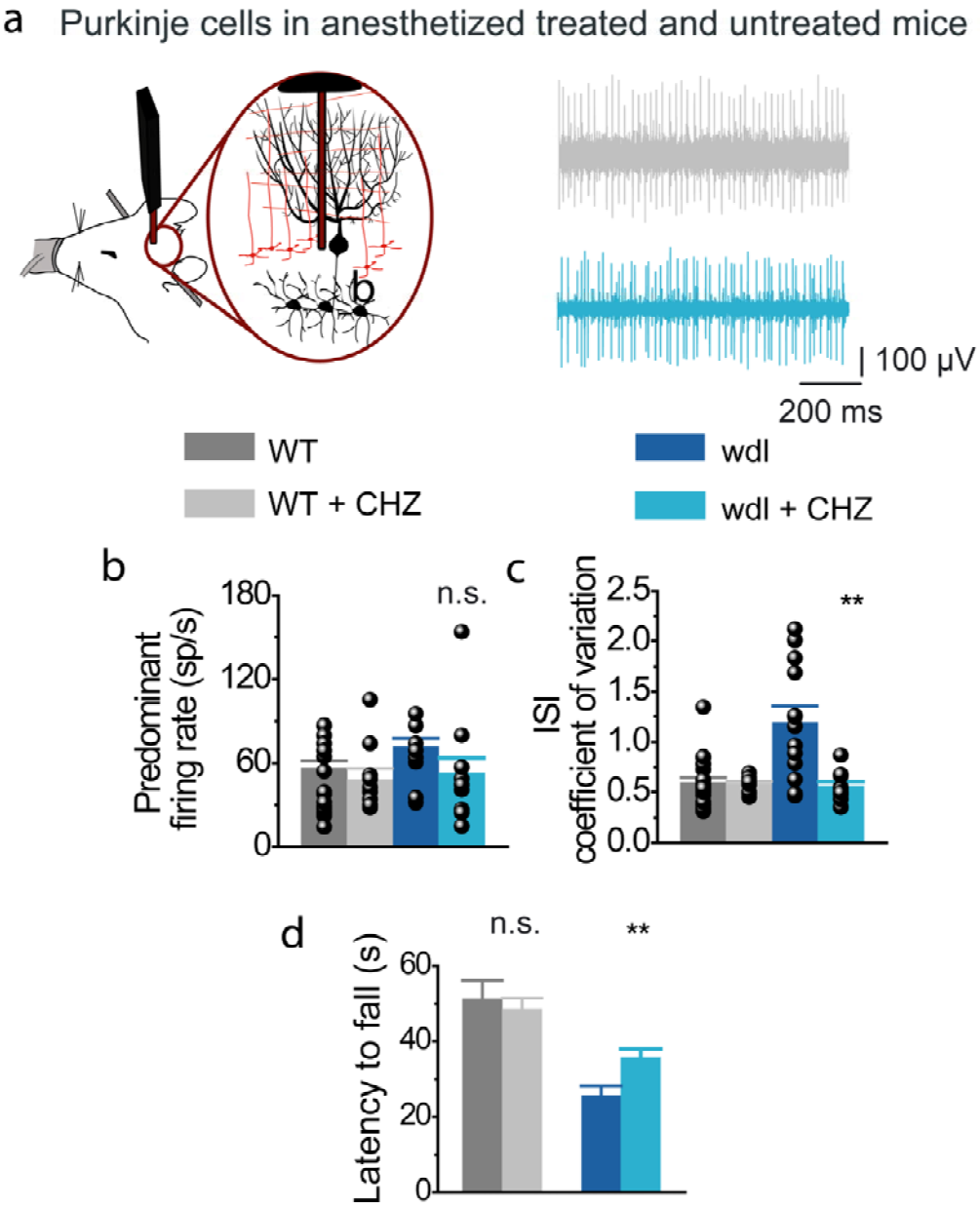
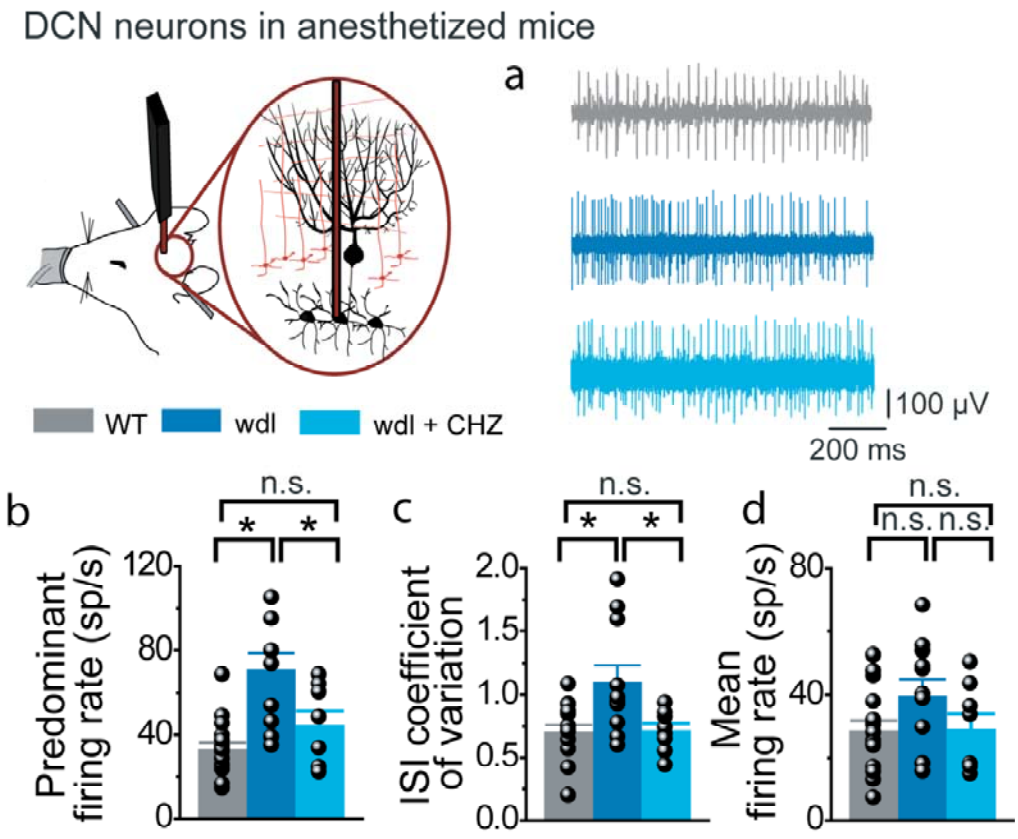

a
Purkinje cells in anesthetized treated and untreated mice
b
100 µV
200 ms
WT
WT + CHZ
wdl
wdl + CHZ
b
Predominant firing rate (sp/s)
180
120
60
0
n.s.
c
ISI coefficient of variation
2.5
2.0
1.5
1.0
0.5
0.0
**
d
Latency to fall (s)
60
40
20
0
n.s.
**

FIGURE 4

Oral administration of chlorzoxazone restores regularity of cerebellar output in anesthetized *waddles* mice.

(a) Typical behavior of DCN neurons recorded in untreated anesthetized wild type and *waddles* mice before and after treatment with CHZ. Individual and average ± S.E.M. values for **(b)** the predominant firing rate, **(c)** coefficient of variation and **(d)** mean firing rate of DCN neurons


DCN neurons in anesthetized mice
a
WT
wdl
wdl + CHZ
100 μV
200 ms
b
Predominant firing rate (sp/s)
120
80
40
0
n.s.
*
*
c
ISI coefficient of variation
2.0
1.5
1.0
0.5
0.0
n.s.
*
*
d
Mean firing rate (sp/s)
80
40
0
n.s.
n.s. n.s.

CHAPTER VI:

A CONTINUUM IN ABERRANT CEREBELLAR OUTPUT PATTERNS CAN CAUSE A SPECTRUM OF MOTOR SYMPTOMS

Esra Tara and Kamran Khodakhah

ABSTRACT

The identification of genetic defects underlying a majority of inherited forms of ataxia implicates a wide range of ubiquitous cellular pathways in its pathomechanism. Nevertheless the phenotype in affected individuals ultimately involves a loss of motor coordination either alone ("pure" cerebellar syndromes) or accompanied by a set of additional motor symptoms such as dystonia, dyskinesia or chorea ("cerebellar ataxia plus" syndromes). We compared the cerebellar output patterns in mutant mice, each of which modeling a different inherited cerebellar disorder by virtue of its unique genetic mutation. We found similar alterations in cerebellar output patterns to give rise to a spectrum of motor dysfunction ranging in severity from ataxia to dystonia.

INTRODUCTION

Inherited cerebellar syndromes arise from a wide variety of genetic mutations. Likely due to the diversity of the altered gene products, the link between dysfunction at the cellular level and the resulting motor symptoms is poorly understood, however many of the altered protein products play important roles in the proper functioning of cerebellar neurons, with Purkinje cells being a common site of dysfunction.

In Chapter II, it was found that in a mouse model of episodic ataxia type 2 (EA2) exhibiting a spectrum of motor dysfunction that includes ataxia of varying severity, dyskinesia and dystonia, the extent of erratic firing or noise in the cerebellar output predicted the severity of the symptoms. Thus, moderate ataxia correlated with an erratic output from the cerebellum whereas severe dystonia was accompanied by high frequency burst firing. Cerebellar output was found to be similarly aberrant in the kelch-like 1 knockout (*klh1*$^{-/-}$) mouse model of spinocerebellar ataxia 8 (SCA8) examined in Chapter 4, as well as the *waddles* mutant mouse in Chapter 5.

Prior studies indicate that the targeted deletion of the *klchl1* gene in Purkinje cells is sufficient to mimic the phenotype of human SCA8 patients (He et al., 2006), and the Purkinje cell-specific expression of the *tottering* mutation recapitulates every aspect of its motor symptoms (Mark et al., 2011). Similarly, the *waddles* mouse carries a mutation in the gene encoding the carbonic anhydrase-related protein VIII (car8), which is highly and almost exclusively expressed in Purkinje cells (Kato, 1990; Jiao et al., 2005). Thus, in all these mice there is evidence implicating the cerebellum as the site of pathogenesis. Although the phenotype of each mouse results from mutations in a variety of genes, all express different combinations of the same set of symptoms. The *klch1*$^{-/-}$ mouse has a

mild form of ataxia (He et al., 2006), the *tottering* exhibits two phenotypes, a moderate ataxia that is present all the time (baseline phenotype) interrupted by dyskinesia episodes during which its symptoms progress in severity from severe ataxia, to dyskinesia and dystonia at the peak severity of an attack (Fureman et al., 2002; Alvina and Khodakhah, 2010; Scholle et al., 2010). The phenotype of *waddles* is characterized by moderate ataxia apparent in its wobbly side-to-side gait and poor performance on the rotarod (Jiao et al., 2005). The *waddles* mouse additionally exhibits several characteristic signs of dystonia such as Straub tail, an abnormally elevated trunk and co-contraction of limb flexors and extensors (appendicular dystonia) exacerbated upon ambulation (Jinnah and Hess, 2004; Jiao et al., 2005).

Evidence from the previous chapters indicates that cerebellar output is altered in each of these mouse models, suggesting that erratic cerebellar output is a downstream outcome of a wide variety of mutations affecting the cerebellum. We show here that the nature and severity of the mutants' symptoms correlate with the extent of erratic firing in cerebellar output neurons and provide a mechanism by which both ataxia and dystonia could be expressed as part of a single syndrome. We conclude that erratic cerebellar output may provide a pathomechanism that translates the deleterious effects of a range of genetic deficits into a spectrum of motor dysfunction delimited on one end by ataxia and on the other by dystonia.

METHODS

In vivo electrophysiology in awake freely behaving mice: Mice were prepared for chronic recordings under isoflurane anesthesia. A recording chamber 3 mm in diameter was drilled in the skull above the cerebellum. A microdrive fitted with a single recording electrode (Thomas Recording GmbH) was fixed onto the skull with two bone screws (Plastics One Inc.) and dental cement (M&S Dental Supply). Following surgery, mice were allowed to recover for one week prior to the recording sessions.

Neural signals were band pass filtered (80 Hz – 20 kHz), amplified (2000×) and digitized (20 kHz) using a data acquisition card (PCI-MIO-16XE) and an in house written software based on LabView (National Instruments, Austin, TX). Waveforms were sorted using Offline Sorter software (Plexon Inc, Dallas, TX USA). Only sorted spikes that could be unambiguously identified to be from a single neuron were included in the analysis.

Disability rating scale: The severity of motor dysfunction was quantified according to a previously published scale (Weisz et al., 2005) as follows: 0 = normal motor behavior, 1 = slightly slowed or abnormal movements, 2 = mild impairments, limited ambulation unless disturbed, 3 = moderate impairment, limited ambulation even when disturbed, frequent abnormal postures, 4 = severe impairment, almost no ambulation, sustained abnormal postures, 5 = prolonged immobility in abnormal postures.

RESULTS

The extent of change in cerebellar output correlates with the severity of motor deficits

The symptoms of *klh1*$^{-/-}$, *waddles* and *tottering* can be qualitatively ranked such that the *klh1*$^{-/-}$ has the mildest phenotype, followed by *waddles* and *tottering* in the absence of a dyskinesia (referred to as *tottering* ataxia or ataxic *tottering*) attack and finally *tottering* during an attack (referred to as *tottering* dystonia or dystonic *tottering*). A comparison of the DCN neuron mean predominant firing rate of mutants to that of wild types obtained by combining average values from age- and strain-matched controls yields a similar ranking (mean predominant firing rate wild type: 56 ± 4 spikes/s, n = 44, N = 4; *klh1*$^{-/-}$: 50.2 ± 3.6 spikes/s, n = 24, N = 5; *tottering* (no attack): 69 ± 4 spikes/s, n = 10, N = 4; *waddles*: 71 ± 8 spikes/s, n = 11, N = 3; *tottering* (attack): 86 ± spikes/s, n = 14, N = 4; Figure 1a). The same is true for the coefficient of variation of interspike intervals (wild type: 0.67 ± 0.03; *klh1*$^{-/-}$: 0.88 ± 0.07; *tottering* (no attack): 0.87 ± 0.06; *waddles*: 1.1 ± 0.1; *tottering* (dystonia): 1.516 ± 0.4; Figure 1b). This observation prompted us to examine the relationship between the nuclear neuron firing pattern in each mouse and the severity of the associated symptoms.

Motor dysfunction in mice can be ascertained using several tests. However, quantitatively comparing the disability of these mutant mice to each other is challenging due to the wide spectrum of motor deficits they collectively exhibit. For example, one of the most sensitive tests of motor incoordination is the accelerating rotarod, which can detect subtle motor deficits that often cannot be visually identified, as is the case for the *klh1*$^{-/-}$ mouse (He et al., 2006). On the other hand, it precludes the assessment of severe

motor symptoms, for example during the dyskinesia episodes of the *tottering* mouse, which prevent it from walking (Scholle et al., 2010). In order to compare a spectrum of motor dysfunction that ranges from ataxia to dystonia, we took advantage of a previously published scale that allows observers blinded to the phenotype of the mice to unambiguously score the severity of motor dysfunction by watching videos of the mice in open field (Fureman and Hess, 2005; Alvina and Khodakhah, 2010). The disadvantage of this method is that it precluded the quantitative description of the *klhl1*$^{-/-}$ phenotype which is too mild to detect by eye. The average scores each mutant received described the following ranking. The peak of the *tottering*'s attack scored highest, the *waddles* mouse's phenotype was scored as less severe and the *tottering* in the absence of an attack had the mildest phenotype (mean disability scores of *tottering* (ataxia): 1.6 ± 0.1, n = 6, N = 4; *waddles*: 2 ± 0.2, N = 4; *tottering* (dystonia): 4.25 ± 0.2, n = 6, N = 4; Figure 1c).

In the mutant mice, the DCN pattern of firing recorded in the awake, head-restrained preparation is characterized by a significantly higher CV of ISIs and predominant firing rate compared to wild type cells obtained under the same recording conditions. We predicted that if these changes underlie the expression of cerebellar symptoms, then the extent of erratic firing should tightly correlate with the severity of symptoms. Plotting the mean predominant firing rate (Figure 2 b, d) and mean CV of ISIs (Figure 2 c, d) with respect to the motor disability scores confirmed the presence of such a correlation (predominant firing rate *versus* motor disability $R = 0.98$, $p < 0.05$; CV of ISI *versus* motor disability $R = 0.97$, $p < 0.05$).

The mutant output patterns are not observed in normal mice under physiologically relevant conditions

It is well known that the firing rate of DCN neurons recorded during the performance of various motor tasks modulate in conjunction with multiple movement parameters (Thach, 1970, 1978), which would similarly alter the distribution of interspike intervals and result in a high CV of ISIs. To pinpoint the features of the firing pattern that correlate with motor dysfunction as opposed to motor function, we recorded the activity of DCN neurons in wild type mice as they performed a variety of tasks thought to engage the cerebellum (Fortier et al., 1987; Le Marec et al., 1997). Thus, we calculated the same parameters for cerebellar activity recorded when the mice were 1) freely standing on a flat surface (Figure 3a), 2) head-restrained (Figure 3b), 3) balancing on a beam (Figure 3c) and 4) walking on a treadmill (Figure 3d).

The firing patterns typically associated with each condition are shown in Figure 3. As expected, we found these to be modulated in conjunction with motor behavior, which was reflected as increases in the CV of ISIs (standing: 0.64 ± 0.07, n = 6, N = 1; head-restrained: 0.67 ± 0.03 ±, n = 44; N = 4; balancing: 0.75 ± 0.1, n = 10, N = 2; walking: 1.4 ± 0.1, n = 6, N = 1; Figure 3f). Similarly, we found the predominant firing rate to be lowest when wild type mice were standing, and highest as they walked on the treadmill (standing: 30 ± 4 spikes/s; head-restrained: 56 ± 4 spikes/s; balancing: 64 ± 6 spikes/s; walking: 84 ± 9 spikes/s; Figure 3e). The CV of ISIs obtained from a wild type mouse walking on a treadmill was significantly higher than that obtained from wild types that were either standing, head-restrained or balancing ($p < 0.001$ walking compared to each of the three other conditions). Similarly, the CV of the wild type as it walked on a

treadmill was significantly higher than that of the head-restrained ataxic *tottering* in the ($p < 0.001$). However, the wild type CV in the walking condition did not differ from head-restrained *waddles* ($p = 0.1$) or head-restrained dystonic *tottering* ($p = 0.9$). The predominant firing rate in the wild type walking condition was also significantly higher than the head-restrained ataxic *tottering* ($p < 0.05$) but not from head-restrained *waddles* ($p = 0.3$) or dystonic *tottering* ($p = 0.9$).

To further assess the characteristics of DCN firing in the different mutants and conditions, we generated autocorrelograms for each wild type condition and each mutant We found autocorrelograms of mutants to be characterized by distinct peaks, which did not occur in wild types under any of the conditions (Figure 4).

DISCUSSION

The wide range of mutated genes implicated in hereditary ataxias suggests that multiple pathways can induce motor deficits. The mechanism by which a large group of mutations ultimately impair cerebellar function is by inducing neuronal loss (Klockgether and Paulson, 2011). Another set of mutations either do not associate with significant cerebellar degeneration, or they do so only after the onset of symptoms, indicating that neuronal loss is another victim, rather then a culprit in the motor deficits (Manto and Marmolino, 2009). In chapters 2, 4 and 5, the activity of cerebellar output neurons was found to be markedly altered in ataxic mice whose mutations seemingly have little in common. Moreover, administration of therapeutic agents that alleviated the mice's symptoms concomitantly abolished the changes in cerebellar output.

We took advantage of the spectrum of motor dysfunction collectively expressed by the mutants studied throughout this thesis and found the extent of erratic DCN firing to correlate with the severity of the mutants' symptoms, which spanned from mild ataxia to severe dystonia. Here we propose that within the framework of the spectrum of motor dysfunction studied, ataxia and dystonia are continuous phenomenon, however two distinct mechanisms explain their expression.

A decreased signal-to-noise ratio as a common mechanism

The cerebellum provides the timing signals that enable the contraction of muscles involved in the execution of a given movement to be properly coordinated (Ito, 1984). These signals are thought to be encoded as changes in the rate and pattern of its neurons' firing, and are ultimately relayed out of the cerebellum via the deep cerebellar nuclei (Ito, 1984). To cause ataxia, dysfunction of the cerebellum must ultimately translate into

altered cerebellar output. The absence of a cerebellar output, for example when DCN neurons are degenerated or silenced, would be equivalent to cerebellectomy and would cause, at least acutely, severe ataxia (Holmes, 1917). However a multitude of other alterations within the cerebellar circuitry and/or physiology could result in aberrant cerebellar outputs with the same consequence for motor coordination as the absence of cerebellar output. To compose their activity DCN neurons integrate information conveyed from the computational circuitry of the cerebellar cortex by Purkinje cells with those arriving *via* the excitatory climbing and mossy fibers from olivo-, spino- and cortico-cerebellar fibers. In principle, alterations in any of these components could result in a cerebellar output that is erroneous, contains less information, or has reduced signal-to-noise ratio. Consider, for example, the fact that at the very least tens of Purkinje cells converge onto individual DCN neurons (Person and Raman, 2012). This convergence might allow DCN neurons to extract the common information that is encoded in the activity of the population of the converging Purkinje cells and disregard the noise inherent in their individual activity (Eccles, 1973; Walter and Khodakhah, 2009). A reduction in the number of Purkinje cells, perhaps by their degeneration or silencing, would reduce the number of converging Purkinje cells and the quality of the "averaging" performed by DCN neurons thus degrading their information content. In many instances the disease process might also affect the activity of the Purkinje cells and degrade the quality of information that they encode and relay to DCN neurons. Additionally alterations in the intrinsic properties of DCN neurons, or their synaptic inputs, might be a source of information loss or introduction of additional noise with the same outcome that the signal-to-noise ratio of the information encoded in their activity is reduced.

Erroneous cerebellar output in dyskinesia and dystonia

We propose that the expression of motor symptoms, ataxia, dyskinesia and dystonia depends on the extent of erratic firing at the level of DCN neurons. While a moderate increase in the noise results in ataxia, a more severe increase leads to a gain of malfunction, generating a faulty cerebellar output and giving rise to dyskinesia and finally dystonia.

In wild types walking on a treadmill, the firing of DCN neurons was extensively modulated as indicated by a high predominant firing rate and CV of ISIs. In fact, these two parameters were significantly higher in walking wild types than head-restrained ataxic mice (ataxic *tottering*, *klh1*$^{-/-}$). On the other hand, the characteristics of the wild type DCN firing in the walking condition did not differ from head-restrained dystonic mice (*waddles,* dystonic *tottering*). We propose that the aberrancy of the firing pattern associated with dystonia arises from the fact that the spiking pattern is as variable in head-restrained mutant mice as walking wild types, even though the barrage of inputs modulating the firing pattern associated with walking is entirely absent. This indicates that strong modulation of the cell's output is pathologically generated in a manner that is intrinsic to the neuron.

Extensive evidence indicates that the cerebellum is physically able to generate movements, even if it does not do so under normal conditions. Thus, electrical, chemical and mechanical stimulations of the cerebellum, all conditions that would presumably instigate high frequency firing of a large group of neurons (Brookhart et al., 1950), result in the production of various movements in an impressive number of species ranging from cats to humans (Brookhart et al., 1950; Bremer and Bonnet, 1953; Nashold and

Slaughter, 1969). Additionally, in Chapter 2 it was found that optogenetic stimulation of the deep cerebellar nuclei results in movement as well. Collectively these studies indicate that the cerebellum has the necessary connections such that a strong enough output signal will be interpreted as a command signal at downstream regions.

Erratic cerebellar output as a shared downstream target of mutations

The proposed pathomechanism would be consistent with the finding that bursting activity reliably accompanies dystonia here as well as in the dystonic rat (LeDoux et al., 1998). Burst firing of Purkinje cells has been associated with involuntary eye movements abolished after flocculectomy (Yoshida et al., 2004) and has even been reported in DCN recordings from human dystonia patients (Slaughter et al., 1970). It would also provide an explanation for the observation that eliminating cerebellar output reliably alleviates dyskinesia and dystonia in rodents (LeDoux et al., 1993; Devanagondi et al., 2007), as well as in humans (Heimburger, 1967; Zervas et al., 1967; Fraioli and Guidetti, 1975).

Collectively this evidence suggests that the genetic heterogeneity characteristic of this family of disorders might result in variability not with regard to the nature of the motor symptoms in affected individuals (as far as can be assessed visually) but rather to the severity of a relatively small set of similar symptoms. Such a possibility is in line with the observation that in different *cacna1a* mutants the extent of decrease in P/Q-type calcium current density correlates with the severity of the ensuing motor phenotype, with small decreases causing moderate ataxia (Zwingman et al., 2001), and the complete loss of protein function in the *cacna1a* null mutant resulting in severe dystonia (Fletcher et al., 2001). Similarly in humans large genomic deletions involving *CACNA1A,* which presumably would result in more significant alterations in the gene product, seem to

correlate with more severe motor dysfunction compared to nonsense mutations (Wan et al., 2011). The fact that a change in the physiology of cerebellar neurons can give rise to motor symptoms such as ataxia, dyskinesia and dystonia has been shown numerous times with different experimental approaches (Pizoli et al., 2002; Shakkottai et al., 2004; Walter and Khodakhah, 2006; Neychev et al., 2008; Calderon et al., 2011; Alvarez-Fischer et al., 2012; Fan et al., 2012), all of which would be predicted to have deleterious consequences on the physiology of Purkinje and DCN neurons.

In Chapters 2-5, we found the same drug, chlorzoxazone to alleviate the symptoms of all three mutants. Given the complexity of the disease mechanism and the fact that the functional consequences of mutations are often unknown, identifying overarching trends in the pathomechanism of cerebellar disease and common downstream targets may be useful strategy.

REFERENCES

Alvarez-Fischer D, Grundmann M, Lu L, Samans B, Fritsch B, Moller JC, Schaefer MK, Hartmann A, Oertel WH, Bandmann O (2012) Prolonged generalized dystonia after chronic cerebellar application of kainic acid. Brain research 1464:82-88.

Alvina K, Khodakhah K (2010) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Bremer F, Bonnet V (1953) Action de la strychnine sur les résponses sensorielles et sur les potentiels électriques spotanés de l'écorce cérébelleuse: L'activité convulsive du cervelet. Folia psychiat, neurol et neurochir néerl 56:438-446.

Brookhart JM, Moruzzi G, Snider RS (1950) Spike discharges of single units in the cerebellar cortex. J Neurophysiol 13:465-486.

Calderon DP, Fremont R, Kraenzlin F, Khodakhah K (2011) The neural substrates of rapid-onset Dystonia-Parkinsonism. Nature neuroscience 14:357-365.

Devanagondi R, Egami K, LeDoux MS, Hess EJ, Jinnah HA (2007) Neuroanatomical substrates for paroxysmal dyskinesia in lethargic mice. Neurobiology of disease 27:249-257.

Eccles JC (1973) The cerebellum as a computer: patterns in space and time. The Journal of physiology 229:1-32.

Fan X, Hughes KE, Jinnah HA, Hess EJ (2012) Selective and sustained alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor activation in cerebellum induces dystonia in mice. The Journal of pharmacology and experimental therapeutics 340:733-741.

Fletcher CF, Tottene A, Lennon VA, Wilson SM, Dubel SJ, Paylor R, Hosford DA, Tessarollo L, McEnery MW, Pietrobon D, Copeland NG, Jenkins NA (2001) Dystonia and cerebellar atrophy in Cacna1a null mice lacking P/Q calcium channel activity. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15:1288-1290.

Fortier PA, Smith AM, Rossignol S (1987) Locomotor deficits in the mutant mouse, Lurcher. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 66:271-286.

Fraioli B, Guidetti (1975) Effects of stereotactic lesions of the dentate nucleus of the cerebellum in man. Applied neurophysiology 38:81-90.

Fureman BE, Hess EJ (2005) Noradrenergic blockade prevents attacks in a model of episodic dysfunction caused by a channelopathy. Neurobiology of disease 20:227-232.

Fureman BE, Jinnah HA, Hess EJ (2002) Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. Pharmacology, biochemistry, and behavior 73:631-637.

He Y, Zu T, Benzow KA, Orr HT, Clark HB, Koob MD (2006) Targeted deletion of a single Sca8 ataxia locus allele in mice causes abnormal gait, progressive loss of motor coordination, and Purkinje cell dendritic deficits. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:9975-9982.

Heimburger RF (1967) Dentatectomy in the treatment of dyskinetic disorders. Confinia neurologica 29:101-106.

Holmes G (1917) The symptoms of acute cerebellar injuries due to gunshot injuries. Brain : a journal of neurology 40:461-535.

Ito M (1984) The Cerebellum and Neural Control. New York: Raven.

Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, Gu W (2005) Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. Genetics 171:1239-1246.

Jinnah HA, Hess EJ (2004) Animal Models of Movement Disorders. San Diego: Elsevier.

Kato K (1990) Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. FEBS letters 271:137-140.

Klockgether T, Paulson H (2011) Milestones in ataxia. Movement disorders : official journal of the Movement Disorder Society 26:1134-1141.

Le Marec N, Caston J, Lalonde R (1997) Impaired motor skills on static and mobile beams in lurcher mutant mice. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 116:131-138.

LeDoux MS, Lorden JF, Ervin JM (1993) Cerebellectomy eliminates the motor syndrome of the genetically dystonic rat. Experimental neurology 120:302-310.

LeDoux MS, Hurst DC, Lorden JF (1998) Single-unit activity of cerebellar nuclear cells in the awake genetically dystonic rat. Neuroscience 86:533-545.

Manto M, Marmolino D (2009) Cerebellar ataxias. Current opinion in neurology 22:419-429.

Mark MD, Maejima T, Kuckelsberg D, Yoo JW, Hyde RA, Shah V, Gutierrez D, Moreno RL, Kruse W, Noebels JL, Herlitze S (2011) Delayed postnatal loss of P/Q-type calcium channels recapitulates the absence epilepsy, dyskinesia, and ataxia phenotypes of genomic Cacna1a mutations. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4311-4326.

Nashold BS, Jr., Slaughter DG (1969) Effects of stimulating or destroying the deep cerebellar regions in man. Journal of neurosurgery 31:172-186.

Neychev VK, Fan X, Mitev VI, Hess EJ, Jinnah HA (2008) The basal ganglia and cerebellum interact in the expression of dystonic movement. Brain : a journal of neurology 131:2499-2509.

Person AL, Raman IM (2012) Purkinje neuron synchrony elicits time-locked spiking in the cerebellar nuclei. Nature 481:502-505.

Pizoli CE, Jinnah HA, Billingsley ML, Hess EJ (2002) Abnormal cerebellar signaling induces dystonia in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7825-7833.

Scholle HC, Jinnah HA, Arnold D, Biedermann FH, Faenger B, Grassme R, Hess EJ, Schumann NP (2010) Kinematic and electromyographic tools for characterizing movement disorders in mice. Movement disorders : official journal of the Movement Disorder Society 25:265-274.

Shakkottai VG, Chou CH, Oddo S, Sailer CA, Knaus HG, Gutman GA, Barish ME, LaFerla FM, Chandy KG (2004) Enhanced neuronal excitability in the absence of neurodegeneration induces cerebellar ataxia. The Journal of clinical investigation 113:582-590.

Slaughter DG, Nashold BS, Jr., Somjen GG (1970) Electrical recording with micro- and macroelectrodes from the cerebellum of man. Journal of neurosurgery 33:524-528.

Thach WT (1970) Discharge of cerebellar neurons related to two maintained postures and two prompt movements. I. Nuclear cell output. Journal of neurophysiology 33:527-536.

Thach WT (1978) Correlation of neural discharge with pattern and force of muscular activity, joint position, and direction of intended next movement in motor cortex and cerebellum. Journal of neurophysiology 41:654-676.

Walter JT, Khodakhah K (2006) The linear computational algorithm of cerebellar Purkinje cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:12861-12872.

Walter JT, Khodakhah K (2009) The advantages of linear information processing for cerebellar computation. Proceedings of the National Academy of Sciences of the United States of America 106:4471-4476.

Wan J, Mamsa H, Johnston JL, Spriggs EL, Singer HS, Zee DS, Al-Bayati AR, Baloh RW, Jen JC, Investigators C (2011) Large Genomic Deletions in CACNA1A Cause Episodic Ataxia Type 2. Frontiers in neurology 2:51.

Weisz CJ, Raike RS, Soria-Jasso LE, Hess EJ (2005) Potassium channel blockers inhibit the triggers of attacks in the calcium channel mouse mutant tottering. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4141-4145.

Yoshida T, Katoh A, Ohtsuki G, Mishina M, Hirano T (2004) Oscillating Purkinje neuron activity causing involuntary eye movement in a mutant mouse deficient in the glutamate receptor delta2 subunit. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2440-2448.

Zervas NT, Horner FA, Pickren KS (1967) The treatment of dyskinesia by stereotxic dentatectomy. Confinia neurologica 29:93-100.

Zwingman TA, Neumann PE, Noebels JL, Herrup K (2001) Rocker is a new variant of the voltage-dependent calcium channel gene Cacna1a. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:1169-1178.

FIGURES

FIGURE 1

The activity of DCN neurons is similarly altered in mutant mice exhibiting ataxia, dyskinesia and dystonia.

Average ± S.E.M. values for DCN **(a)** predominant firing rate and **(b)** interspike interval coefficient of variation in the awake, head-restrained wild type, *klhl1*$^{-/-}$, ataxic *tottering*, *waddles* and dystonic *tottering* mutant mice. **(c)** Motor disability scores of wild type, ataxic *tottering, waddles* and dystonic *tottering.*

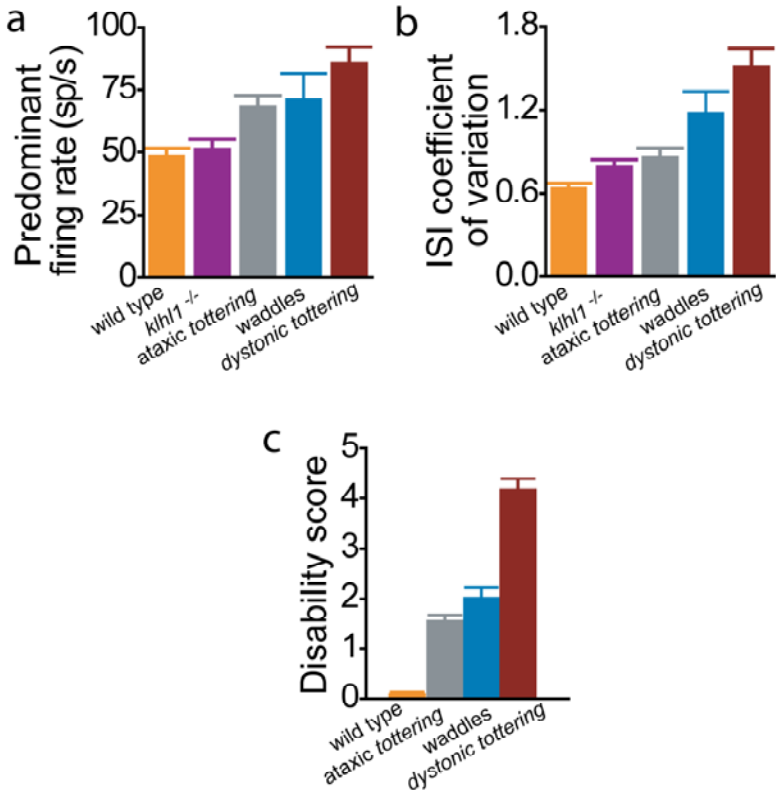

a
Predominant firing rate (sp/s)
100
75
50
25
0
wild type
klhl1 -/-
ataxic tottering
waddles
dystonic tottering
b
ISI coefficient of variation
1.8
1.2
0.6
0.0
wild type
klhl1 -/-
ataxic tottering
waddles
dystonic tottering
c
Disability score
5
4
3
2
1
0
wild type
ataxic tottering
waddles
dystonic tottering

FIGURE 2

The erratic activity of DCN neurons correlates with the motor disability score of wild types and mouse models of inherited cerebellar syndromes exhibiting ataxia, dyskinesia or dystonia.

(a-c) Average ± S.E.M. values for DCN **(a,c)** predominant firing rate and interspike **(b,c)** interval coefficient of variation as a function of motor disability score in wild type and mutant mice.

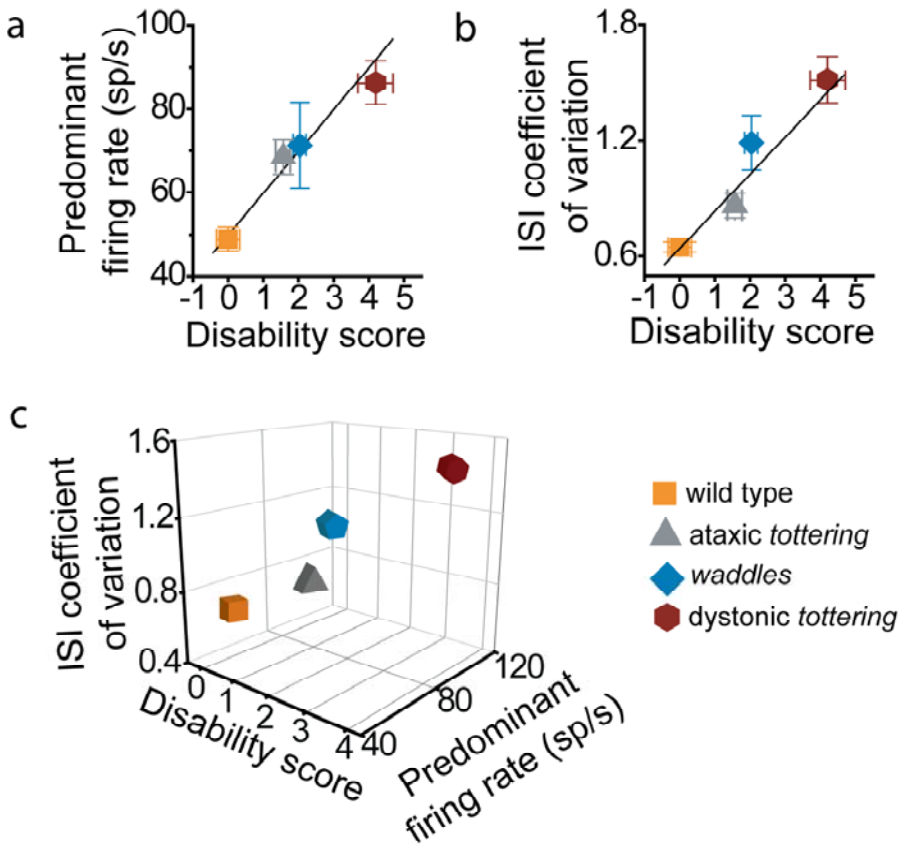

a
Predominant firing rate (sp/s)
100
80
60
40
-1 0 1 2 3 4 5
Disability score
b
ISI coefficient of variation
1.8
1.2
0.6
-1 0 1 2 3 4 5
Disability score
c
ISI coefficient of variation
1.6
1.2
0.8
0.4
0 1 2 3 4
Disability score
40 80 120
Predominant firing rate (sp/s)
wild type
ataxic tottering
waddles
dystonic tottering

FIGURE 3

Wild type DCN neuron activity modulates with the performance of motor tasks.

Typical single unit activity of wild type DCN cells recorded while mice were **(a)** standing still on a flat platform, **(b)** head-restrained, **(c)** balancing on a beam and **(d)** walking on a treadmill.

Average ± S.E.M. values of **(e)** predominant firing rate **(f)** interspike interval coefficient of variation **(g)** mean firing rate and **(h)** interspike interval distribution of wild type DCN neurons for each motor task.

Pink denotes standing still on a platform, orange denotes head-restrained, green denotes balancing on a beam and red denotes walking on a treadmill.

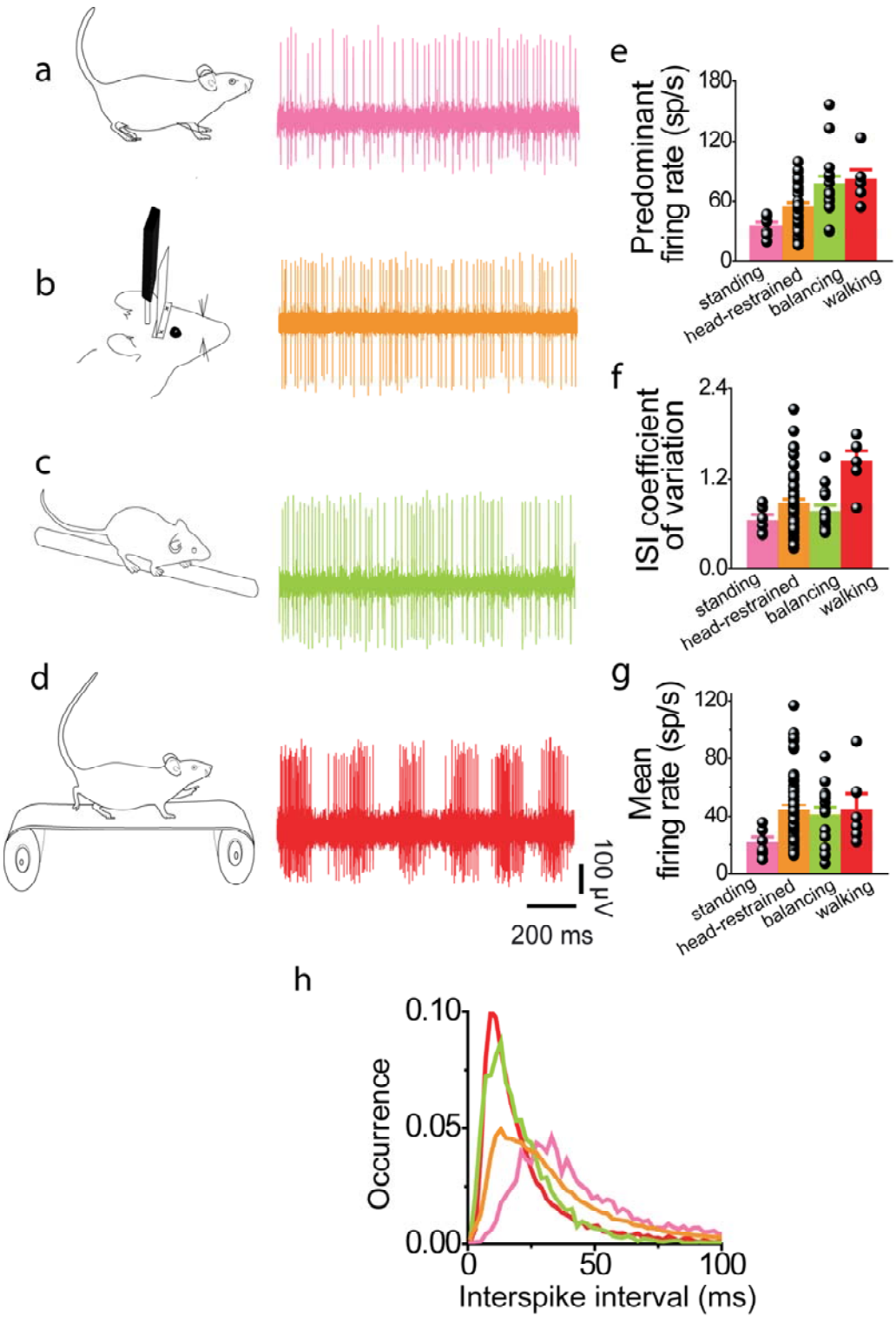
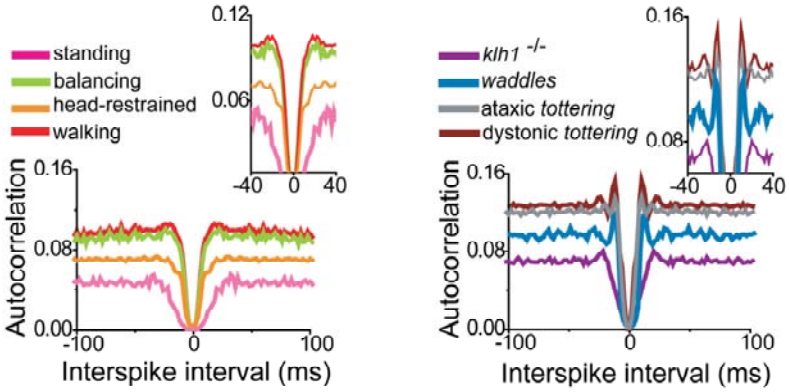

a
b
c
d
100 µV
200 ms
e
Predominant firing rate (sp/s)
180
120
60
0
standing
head-restrained
balancing
walking
f
ISI coefficient of variation
2.4
1.2
0.0
standing
head-restrained
balancing
walking
g
Mean firing rate (sp/s)
120
80
40
0
standing
head-restrained
balancing
walking
h
Occurrence
0.10
0.05
0.00
0
50
100
Interspike interval (ms)

FIGURE 4

Interspike interval autocorrelograms show distinct peaks for mutants but not for wild type DCN neurons.

(a) DCN neuron Interspike interval autocorrelograms for **(a)** wild type mice while standing on a flat platform, head-restrained, balancing on a beam and walking on a treadmill **(b)** *klhl1*$^{-/-}$, ataxic *tottering*, *waddles* and dystonic *tottering* mice.


standing
balancing
head-restrained
walking
0.12
0.06
-40
0
40
0.16
0.08
0.00
Autocorrelation
-100
0
100
Interspike interval (ms)
klh1 -/-
waddles
ataxic tottering
dystonic tottering
0.16
0.08
-40
0
40
0.16
0.08
0.00
Autocorrelation
-100
0
100
Interspike interval (ms)

-

CHAPTER VII:

DISCUSSION

OVERVIEW

The overall goal of this thesis was to delineate the output patterns of the cerebellum that are associated with its dysfunction. As described in detail throughout, growing clinical evidence implicates the cerebellum as the site of pathogenesis in several forms of ataxia, dyskinesia and dystonia. This evidence provides the basis for the studies undertaken and described in Chapters 2 through 5. Chapter 2 begins by asking whether the expression of motor symptoms correlates with changes in the firing pattern of cerebellar output neurons. For this the spectrum of motor dysfunction expressed by the *tottering* mouse was chosen as a model system for two major advantages it provides. First, the pathological signals that mediate the dyskinesia and dystonia symptoms are thought to be cerebellar in origin, as removing the cerebellum abolishes (Neychev et al., 2008) and targeted expression of its mutation in Purkinje cells replicates them (Mark et al., 2011). Second, the *tottering* exhibits a combination of ataxia, dyskinesia and dystonia (Jinnah and Hess, 2004; Scholle et al., 2010), which allowed us to examine the pathophysiology that leads to the expression of one symptom *versus* the other. This study showed that an erratic DCN firing pattern correlates with the occurrence of symptoms. The pattern of firing changed from slightly irregular to strong bursting as the symptoms progressed from ataxia to dystonia. This change in nuclear neuron behavior could form the basis for the erroneous cerebellar output causing abnormal muscle contractions, as indicated by the cessation of symptoms upon regularizing it in the *tottering*, and their expression upon mimicking it in wild types.

From the observation that cerebellectomy alleviated *tottering* and other dystonic mice's symptoms (LeDoux et al., 1993; Devanagondi et al., 2007), a putative link

between aberrant cerebellar output and the expression of motor symptoms had previously been established. However, it was not until the experiments undertaken in Chapter 2 that the nature of the proposed "aberrant" activity could be identified. Moreover, the results in Chapter 2 provided a plausible mechanism that could reconcile the seemingly contradictory finding that ataxia, a loss of cerebellar function, and dystonia, presumably a gain in cerebellar malfunction, could co-exist.

Establishing a physiological basis for the *tottering*'s dyskinesia attacks allowed us to further examine possible signaling pathways which could account for stressful stimuli triggering motor symptoms, a feature that is unique to the EAs among inherited ataxias. A putative link between attacks and noradrenergic signaling had previously been put forth based upon the finding that systemic blockade of α noradrenergic receptors abolished stress-induced attacks (Fureman and Hess, 2005). This finding led us to investigate a plausible mechanism that could account for norepinephrine's apparent deleterious effect on *tottering*'s motor behavior. Based upon the burst firing of Purkinje neurons observed in Chapter 2 as well as the therapeutic effects of K_{Ca} channel activators on *tottering*'s motor symptoms observed in studies here and prior (Walter et al., 2006; Alvina and Khodakhah, 2010b), a signaling pathway that could lead an increased norepinephrine release onto the cerebellum to cause burst firing was postulated and tested.

Taken together, the results from Chapters 2 and 3 delineate a mechanism whereby a stress-induced increase of norepinephrine release from the locus ceruleus onto the

cerebellum would initiate burst firing in Purkinje cells. In turn, this altered output from the cerebellar cortex would instigate a change in the firing behavior of DCN neurons and form the basis for the erroneous signals mediating abnormal muscle contractions during *tottering*'s attacks. Moreover, the degradation of DCN firing pattern from irregular to burst firing would determine the progression of *tottering*'s symptoms from ataxia to dystonia. These findings combined with additional key pieces of evidence from the literature implicating Purkinje cell physiology as a potential target of various mutations in seemingly unrelated genes, motivated the studies undertaken in the subsequent chapters. In Chapter 4, the molecular underpinnings of the motor dysfunction in a spinocerebellar ataxia were examined. A crucial difference between EAs and SCAs is that in most SCAs, it is assumed that it is the degeneration of Purkinje cells that instigates the slowly progressing motor deficits. The absence of cell death in the *klhl1*$^{-/-}$ mouse, whose phenotype mimics that of SCA8 patients, provided the opportunity to test the hypothesis that it is ultimately a similar dysfunction in cerebellar neurons rather than a reduction in their number that underlies the motor symptoms. The data presented in Chapter 4 indicate that in an attempt to compensate for the genetic mutation's immediate effects on the excitability of Purkinje cells, a circuit level compensatory mechanism ultimately gives rise to similar erratic Purkinje cell firing associated with the motor symptoms of the EA mouse model *tottering.* Such a change in Purkinje cell activity drives cerebellar output neurons to fire erratically in the *tottering* and *klhl1*$^{-/-}$ mice, and further evidence in Chapters 2 through 5 indicate that pharmacologically regularizing this activity alleviates the motor symptoms in both the EA as well as the SCA models.

While Chapters 2 through 5 consistently provide evidence for the deleterious effects of disrupting cerebellar output on motor function, Chapter 6 uses these findings to first test their general applicability to inherited cerebellar disease and to characterize the properties distinguishing normal output patterns from abnormal ones. Thus, the results of Chapter 6 are not only important for inherited cerebellar disease, but are of importance for understanding normal cerebellar functioning as well. The analyses performed in Chapter 6 indicate that cerebellar-induced ataxia, dyskinesia and dystonia together form a single spectrum of motor dysfunction, rather than distinct symptoms with unique pathomechanisms. In three mouse models examined throughout this thesis, the extent of the impairment was dependent on the magnitude of erratic neuronal activity. Thus, moderate noise in the baseline activity of DCN neurons associated with a moderately ataxic phenotype, and burst firing manifested as dystonia.

A. MOUSE MODELS OF MOTOR DYSFUNCTION

1. VALIDITY OF THE MOUSE MODELS

To study inherited cerebellar disease, mutant mice modeling them were relied upon. The specific mutants used as part of this research originally resulted from both a reverse genetics approach with the introduction of a known human mutation into the mouse genome (*klhl1*$^{-/-}$) (He et al., 2006) as well as a forward genetics approach whereby mutations arose spontaneously and their identity as well as their association with human syndromes were only subsequently discovered (*tottering*, *waddles*) (Green and Sidman, 1962; Jiao et al., 2005). Nevertheless all three mice faithfully model several aspects of the human disease, keeping in mind that the interpretation of findings is limited by certain differences between the mouse and human syndromes.

The *tottering* mouse has high etiological validity given that it recapitulates both the genetic mutation of human EA2 patients and the consequence of the mutation on the gene product, a decreased P/Q-type calcium current density (Wakamori et al., 1998; Rajakulendran et al., 2010). Moreover, the motor symptoms in the *tottering* mirror those of EA2 patients (Fureman et al., 2002; Jen et al., 2004), and additionally improve in response to the same drugs used for the treatment of EA2 (Weisz et al., 2005; Alvina and Khodakhah, 2010a; Strupp et al., 2011), indicating high face and predictive validities. There are two important features distinguishing *tottering*'s phenotype from that of EA2 in humans. First, the *tottering*'s attacks are in general more severe than human EA2 patients. Likely as a result of the vast number of different mutations that give rise to the syndrome, the severity of its symptoms vary from one person to the next and affected individuals can, but do not necessarily exhibit dyskinesia and dystonia during attacks (Jen et al., 2004). However, the spectrum of symptoms resulting from *CACNA1A* mutations is continuously expanding and it is found to include dystonia and dyskinesia more frequently than ever (Giffin et al., 2002; Spacey et al., 2005; Roubertie et al., 2008; Cuenca-Leon et al., 2009; Blumkin et al., 2010). Second, while in the *tottering* mouse, the baseline ataxia likely results from altered Purkinje cell physiology, in human EA2 patients the baseline phenotype is either not ataxic, or when it is, the onset of ataxia and its progression closely follows that of the degeneration of cerebellar neurons (Denier et al., 1999; Jen et al., 2007). Given that neuronal degeneration is absent in (young) *tottering* mice it is difficult to interpret these results within the specific context of ataxia as part of the EA2 syndrome. It is important for future studies to determine whether or

not cell loss occurs in older *tottering* mutants, given that in humans neurodegeneration generally onsets later in life (Baloh and Jen, 2000; Baloh, 2012). Clearly many inherited forms of ataxia occur in the absence of neuronal degeneration indicating that similar physiological alterations are likely at play.

The genetic basis of SCA8 has recently been subject to controversy and the role of klhl1 in disease pathomechanism was questioned (Moseley et al., 2006). Further studies are most definitely needed to scrutinize both the involvement of klhl1 and the possibility that multiple etiologies play a role rather than a single one to the exclusion of all others. Nevertheless, the *klhl1* knockout model has high face validity, mimicking both the symptoms as well as the morphological changes observed as part of the human SCA8 syndrome (He et al., 2006).

Mutations in the *CA8* gene have only recently been identified as part of a recessively inherited human ataxic syndrome (Turkmen et al., 2009; Kaya et al., 2011; Najmabadi et al., 2011). Clearly the mutation gives rise to severe ataxia in both mice and humans (Jiao et al., 2005; Kaya et al., 2011). However, as a result of the novelty of its characterization and likely infrequent occurrence of this mutation in humans, few affected families have been reported, preventing a reliable comparison of additional aspects of the disease in humans *versus* mice to be made. It is interesting to note that in humans an unvarying feature is the sustained flexion of limb muscles with minimal movement of joints, the same observation that led to the characterization of appendicular dystonia in the *waddles* mouse (Turkmen et al., 2009).

It is well known that results obtained from a single animal model do not always generalize to other animal models or human disease (Jinnah et al., 2005; Jinnah and Hess,

2008). The studies described in this thesis addressed this limitation by demonstrating similar effects in multiple distinct mutant mice. Obtaining analogous results across different mutants increases the likelihood of uncovering more general principles regarding the neural substrates of cerebellar disease.

2. ASSESSMENT OF MOTOR FUNCTION

Inherent to the study of motor disorders in mouse models is the difficulty to assess the severity of dysfunction. The simplest way to assess motor dysfunction (in both humans and mice) is by eyeballing it, as was done in this thesis on numerous occasions. While subjective by design, the rating scales such as the one used in these studies have marked inter-observer reliability, which when combined with blinded-conditions are found to accurately depict the symptoms (Jinnah and Hess, 2005). Although seemingly crude, the use of a disability scale has many advantages. Most importantly, it allows the assessment of disability in different models whose phenotypes collectively represent a wide spectrum of motor dysfunction. Often the severity or subtlety of the symptoms in one mouse precludes it from performing a given motor task or the motor task from detecting its deficit. This is the case for the mild *klhl1*$^{-/-}$ phenotype, which cannot be visually identified, and the severe *tottering* dyskinesia episodes which prevent it from running on a rotarod. On the other hand, two important limitations must be kept in mind while interpreting results. First, the severity scale alone does not allow symptoms to be distinguished as ataxia *versus* dyskinesia or dystonia. The phenotype of each mouse used as part of this study had previously been characterized by means of rigorous motor testing (Jiao et al., 2005; He et al., 2006; Walter et al., 2006), including EMG studies to assess dystonia in the *tottering* mouse (Scholle et al., 2010). Moreover the disability scale has been used to quantify the *tottering*'s symptoms by numerous investigators (Shirley et al., 2008; Alvina and Khodakhah, 2010b), and in fact the scores it received at a specific time during its attacks have been compared to the pattern of muscular activity detected by EMG (Shirley et al., 2008; Scholle et al., 2010). It is based on these studies that

conclusions were drawn regarding ataxia *versus* dystonia or dyskinesia by means of disability scores. On the other hand, while the *waddles* mouse's ataxia has been rigorously quantified using traditional tests of motor function (Jiao et al., 2005), its appendicular dystonia was "diagnosed" by the visual identification of several dystonic symptoms (E.J. Hess personal communication). Although this is how dystonia is characterized in humans, given the potential implications of findings in mouse models on human therapies, a careful, quantitative measure of *waddles*' appendicular dystonia is lacking and certainly needed to unarguably describe its symptoms as dystonia.

Second, by virtue of the discrete nature of the scoring, the results obtained using disability scales are discontinuous. The implication for the studies outlined here is that it is ultimately impossible to determine when ataxia ends and dyskinesia begins, and therefore to quantitatively assess when abnormal cerebellar activity becomes an erroneous signal. However, the results from this thesis support the conclusion that these symptoms are a continuum rather than discrete and distinct deficits. Additional EMG studies are crucial to determine whether this is the case at the muscular level.

B. CHARACTERIZATION OF CEREBELLAR ACTVITIY *IN VIVO*

A significant caveat inherent to the study of cerebellar activity in awake mice is the possibility that the observed activity patterns reflect the extensive feedback the cerebellum receives from muscles rather than deleterious effects of genetic mutations that drive the motor dysfunction. Since blocking all inputs to the cerebellum is experimentally not feasible, it would be impossible to unequivocally prove that a given pattern of Purkinje cell activity represents pathological changes in its physiology rather than

changes in the activity of muscles it receives input from. However, a reasonable amount of evidence corroborates the former possibility. For example, experiments using *in situ* hybridization for *c-fos* messenger RNA to pinpoint the neuroanatomical substrate of *tottering* attacks show that *c-fos* activation in Purkinje and DCN neurons is highest and temporally precedes the expression in the cerebral cortex (Campbell and Hess, 1998). Moreover, for each mouse model used as part of this thesis, substantial evidence suggests a causal link between changes in the excitability of Purkinje cells and the motor symptoms that result from the mutation. For instance it was recently shown that targeted-expression of the *tottering* mutation in Purkinje cells is sufficient to recapitulate all aspects of the *tottering* phenotype (Mark et al., 2011). The phenotype ensuing from the Purkinje cell specific knockout of the *klhl1* gene is indistinguishable in terms of phenotype and brain morphology from its global deletion (He et al., 2006). Although the outcome of a targeted expression of the *waddles* mutation in Purkinje cells is unknown, the expression of the *car8* gene is virtually limited to Purkinje cells (Kato, 1990; Jiao et al., 2005). In Chapter 2, it was found that intracerebellar injections of a K_{Ca} channel activator abolished *tottering*'s attacks. When the activity of Purkinje cells was monitored simultaneously with the intracerebellar injection it was found that they fired much more regularly. Cerebellar-specific perfusion of an agent that induced burst firing of Purkinje cells was sufficient to mimic the *tottering*'s symptoms in wild types. In addition, intracerebellar injections of caffeine or norepinephrine were sufficient to induce dyskinesia in the *tottering*, which provided further evidence for the initiation of

paroxysmal dysfunction in the cerebellum, as well as a plausible mechanism that would lead stress to induce burst firing of Purkinje neurons.

C. CEREBELLAR CONTROL OF MOVEMENT

An understanding of the abnormal nature of cerebellar output signals or the mechanism by which abnormal cerebellar signals lead to motor deficits ultimately requires these properties to be understood in the context of normal cerebellar function. The nature of the cerebellar signals mediating normal motor function is unknown and what cerebellar-generated signals do is not clear either. However, within the context of what is known with regard to cerebellar function, as well as what has been theorized, there exists several clues that corroborate and explain the findings from this thesis.

1. A DECREASED SIGNAL-TO-NOISE RATIO: ATAXIA

At the muscular level, EMG recordings in humans with cerebellar disease indicate that ataxic patients have defects in the pattern of the phasic burst of muscular activity associated with these movements. For example during fast flexions of the arm, the timing of muscular contractions is altered such that the contraction of the antagonist muscle is delayed and its duration is longer, and the normal pause between the contractions of reciprocal muscles is shorter, resulting in excessive co-contraction of agonist and antagonist muscles (Hallett et al., 1975b).

The ISI CV of Purkinje and nuclear neurons in the presence of synaptic blockers *in vitro* is on the order of 5% (Raman et al., 2000; Walter et al., 2006; Alvina and Khodakhah, 2008), in other words an intrinsic activity that is remarkably precise. The exquisite regularity of Purkinje and DCN neuron pacemaking forms the base that allows signals to accurately be encoded as changes in the rate and pattern of their firing (Ito, 1984).

The intrinsic pacemaking of Purkinje cells does not carry any meaningful, time-variant information as demonstrated by the persistence of this activity in the absence of synaptic inputs (Raman and Bean, 1999). Time-variant information is relayed to Purkinje cells from cerebellar afferents which provide it with both sensory and cortical information relating to the execution of a movement (Ito, 1984). Thus, Purkinje cells are informed of the intent to execute a movement, the parameters relating to its execution and the context within which it is executed. Purkinje cells integrate this barrage of inputs with their intrinsic pacemaking and encode the signals required for the appropriate timing of muscle contractions as instantaneous changes in the rate of their spontaneous activity (Ito, 1984; Hoebeek et al., 2005; Walter et al., 2006; Walter and Khodakhah, 2006). Therefore a change in the duration of each interspike interval from the average rate of the intrinsic pacemaking should in principle provide the DCN with time-variant information related to the timing of muscle contractions. As a result, the signal to noise ratio of the averaged time-variant information at the level of the DCN is dependent on the regularity of the pacemaking of each individual Purkinje cell providing input to a given nuclear neuron.

It is known that at every level of signal processing, a certain amount of noise is added to neural signals because of the probabilistic nature of synaptic transmission (Shadlen and Newsome, 1994; Stein et al., 2005). The fact that humans are still able to generate highly precise movements suggests the presence of mechanisms that allow the noise added at each level to be reduced at each level as well. It is thought that neural circuits reduce noise in the output of individual neurons by averaging the activity of numerous neurons so as to extract the relevant information common in the output of all

the neurons and eliminate the variability that is unique to each (Shadlen and Newsome, 1994). Eccles postulated that the cerebellar circuitry is ideally suited for this due to the convergence of many Purkinje cells onto a single DCN neuron (Eccles, 1973).

Given the complexity of limb movements, delineating the exact properties of cerebellum's contribution in mediating these has been challenging. One way cerebellar researchers have attempted to examine cerebellar function is by taking a reductionist approach. Thus, the relationship between the cerebellar output signal and muscle contractions is most often studied in the simple circuitry allowing cerebellar neurons to control eye movements. As described in the introduction, cerebellar lesions give rise to certain elementary deficits in muscular control that differ mainly in the muscle groups that are affected (Holmes, 1917). Several parallels can be drawn between the control of limb muscles and that of ocular muscles. It is well known that electrically stimulating the cerebellum causes both eye and limb movements (Cohen et al., 1965), cerebellar lesions invariably give rise to various deficits in the control of both ocular and limb muscles (Holmes, 1917) and the firing of cerebellar neurons modulate in response to both eye and limb movements (Thach, 1970a; Lisberger and Fuchs, 1978). Purkinje cells involved in the control of eye movements reside in the flocculus and paraflocculus and monosynaptically project to superior and medial vestibular nuclei neurons (Langer et al., 1985). The latter neurons are thus considered equivalent to deep cerebellar nuclear neurons and exhibit similar properties in terms of their firing and modulation (Ito, 1984). Vestibular nuclei neurons in turn monosynaptically connect to motor neurons innervating eye muscles (McMasters et al., 1966). The relative simplicity of this circuit has allowed extensive characterization of Purkinje cell firing properties in relation to simple eye

movements (Ito, 1984). Within this circuit, it is well known that the firing rate of Purkinje cells contains sufficient information to ensure proper contraction of eye muscles (Lisberger and Fuchs, 1978; Shidara et al., 1993).

Purkinje cells in the paraflocculus and flocculus modulate their discharge according to gaze velocity and position during smooth pursuit or passive eye tracking (Lisberger and Fuchs, 1978; Lisberger et al., 1981). When the firing of Purkinje cells are examined in relation to smooth pursuit eye movements, it is found that the variation in the movement of the eye and the variability in the firing of Purkinje cells are very tightly correlated (Medina and Lisberger, 2007). In other words, individual Purkinje cells add little noise to the signal they transmitted, and most of the variability in the movement occurred due to the variability in the firing of neurons in upstream regions (Medina and Lisberger, 2007). This suggests that moderate decrease in the signal-to-noise ratio at the level of Purkinje cells could alter the quality of the signal relayed to downstream regions.

Extensive evidence indicates that any pathological modulation of the precision of intrinsic Purkinje cell pacemaking will reduce the accuracy with which a Purkinje cell encodes the strength and timing of the synaptic inputs it receives. The ataxic mouse *ducky* has a mutation in the gene that encodes the $\alpha_2\delta_2$ subunit of P/Q-type calcium channels (Gao et al., 2000). In the *ducky* mouse, the intrinsic pacemaking of Purkinje cells is also erratic and on the order of 0.17 in the absence of synaptic inputs, similar to *tottering* Purkinje cells whose CV is 0.19 *in vitro* (Walter et al., 2006; Alvina and Khodakhah, 2010b). The same parallel fiber stimulation is equally effective in increasing the firing rate of Purkinje cells in *ducky* and wild type mice. However, the timing of parallel fiber input is less accurately encoded in the firing rate of *ducky* Purkinje cells

(Walter et al., 2006). Thus, when the timing of the spikes that compose the tonic pacemaking is more variable, the parallel fiber-driven increase in the firing rate overlaps with the baseline firing rate, which decreases the information contained in the Purkinje cell's output (Walter et al., 2006). This demonstrates the potential impact of noise in the spontaneous activity of Purkinje cells on signal processing. What is the functional impact of such a decreased signal to noise ratio on motor function? Experimental evidence indicates that such a decrease in the signal to noise ratio does in fact translate to motor dysfunction. The proper execution of compensatory eye movements as part of the vestibulo-ocular reflex depend on the ability of floccular Purkinje cells to precisely encode inputs as changes in the pattern of their firing. Although *tottering* Purkinje cells can properly modulate in response to optokinetic stimulation, they exhibit reduced gain values during the optokinetic and vestibulo-ocular reflexes (Hoebeek et al., 2005). When the correlation between the spiking of Purkinje cells and the eye movements is examined, it is found that the increased noise in the tonic activity of *tottering* Purkinje cells decreases the correlation coefficient between these two parameters (Hoebeek et al., 2005). In other words, the timing of the eye movement is less accurately encoded in the firing rate of *tottering* Purkinje cells (Hoebeek et al., 2005). Thus, when the timing of the spikes that compose the tonic pacemaking is more variable, the eye movement-related increase in the firing rate overlaps with the baseline firing rate. As a result, the predictability of simple spikes in wild types is much more reliable than in the *tottering*. In fact the *tottering*'s gain values are indistinguishable from those of flocculectomized wild types and can be rescued by stimulating floccular Purkinje cells to mimic normal simple spike activity patterns (Hoebeek et al., 2005).

2. A GAIN IN CEREBELLAR MALFUNCTION: DYSKINESIA

The ISI CV and predominant firing rate of DCN neurons obtained from wild types walking on a treadmill indicate that their activity is highly modulated during this task as compared to the other conditions. This is an expected result given that under normal conditions when an animal is walking, mossy fibers provide the cerebellum with input relating to several parameters of this movement (Thach, 1970b; Ito, 1984). This signal modifies the behavior of neurons at downstream motor regions such that a given motor command signal is properly timed. The simplest evidence corroborating this conclusion is the marked decrease in the coordination of movements that accompanies cerebellar lesions (Holmes, 1917).

We propose that the aberrancy of the firing pattern in the dystonic *waddles* and *tottering* arises from the fact that they are as variable in a head-restrained condition, where the barrage of inputs modulating the firing pattern associated with walking is entirely absent. This indicates that strong modulation of the cell's output is pathologically driven. These findings need to ultimately be understood in relation to the aberrant muscle activation patterns that characterize dyskinesia and dystonia. In an impressive set of experiments, the activity of a pair of reciprocal hind limb muscles (vastus lateralis – VL; biceps femoris – BF) were monitored as the *tottering*'s symptoms progressed from severe ataxia, to dyskinesia and dystonia (Scholle et al., 2010). The dyskinesia phase of the attack correlated with unusually high discharge of motor units. Moreover, the EMG signals was characterized by the frequent occurrence of doublets and triplets, which are indicative of myokymia (spontaneous involuntary quivering of a few muscles bundles within a muscle that are insufficient to move a limb about a joint)

(Preston and Shapiro, 2002). As the attack progressed, the duration of the bursts of activity in each muscle increased such that each muscle contracted for prolonged periods of time. During the peak of the attack, the bursts of activity in the two muscles partially overlapped for extended periods of time, indicating their simultaneous contraction (Scholle et al., 2010).

Extensive evidence indicates that the cerebellum is physically able to generate movements, even if it does not do so under normal conditions. Thus, electrical, chemical and mechanical stimulations of the cerebellum, all conditions that would presumably instigate high frequency firing of a large group of neurons (Brookhart et al., 1950), result in the production of various movements in an impressive number of species ranging from cats to humans (Brookhart et al., 1950; Bremer and Bonnet, 1953; Nashold and Slaughter, 1969). Additionally, in Chapter 2 it was found that optogenetic stimulation of the deep cerebellar nuclei results in movement as well. Collectively these studies indicate that the cerebellum has the necessary connections such that a strong enough output signal will be interpreted as a command signal at downstream regions. This could occur for example via the monosynaptic connection between the cerebellum and spinal cord (Asanuma et al., 1980, 1983). Alternatively, cerebellar output signals could alter the behavior of downstream cortical and brainstem motor regions. Either way, the cerebellum is separated from motor neurons by at most a tri-synaptic connection (DCN → thalamus → motor cortex → motor neuron pathway, Asanuma et al. (1974); Thach and Jones (1979)), but there exist several shorter pathways as well (DCN → red nucleus → motor neuron, Allen and Tsukahara (1974)).

It could be argued that a signal ensuing from the cerebellum is not relayed to muscles as a command signals under normal circumstances and therefore would not be under any other conditions either. In fact the exact properties of the signal provided by the cerebellum to motor command centers is unknown. The only known properties of the cerebellum are 1) that it does not translate the "will" to move into action and 2) that it coordinates movements (Flourens, 1824). A movement results from the contraction of muscle. A coordinated movement results from the contraction of a set of muscles at the right time (Hallett et al., 1975a, b). It is thought that the cerebellar output signal contains information that determines what that "right time" is. *Ipso facto*, the cerebellar signal must influence a given motor command signal in a certain way such that it reaches muscles at the correct time.

If the firing rate of Purkinje cells were modulated due to pathological factors (perhaps due to a decrease in the activity of SK channels caused by stress) would downstream targets (for example vestibular nuclear neurons and ocular motor neurons) interpret this signal as a command signal and instigate abnormal muscle contractions? At least within the simple circuitry allowing Purkinje cells to control the contraction of ocular muscles this seems to be the case. Thus, in a previous study the motor performance of mouse mutants deficient in the cerebellar-specific δ2 subunit of glutamate (Yoshida et al., 2004) receptors ($\delta 2^{-/-}$) was compared to that of mice lacking Purkinje cells all together (*Lurcher* mutant) and wild types (Yoshida et al., 2004). It was found that the $\delta 2^{-/-}$ mutant performed markedly worse on the rotarod test of motor coordination than the mouse lacking Purkinje cells. The motor disability was further characterized by measuring the eye movements of the mice, which indicated that the $\delta 2^{-/-}$ exhibited

excessive involuntary eye movements whereas the Purkinje cell deficient mouse did not (Yoshida et al., 2004). Moreover, removing the region of the cerebellum that controls ocular muscles (flocculus) alleviated the hyperkinetic movements (Yoshida et al., 2004). Finally, Purkinje cells in the $\delta 2^{-/-}$ mutant were found to fire bursts of action potentials at high frequency separated by pauses (Yoshida et al., 2004). These results corroborate ours, which predict that the motor dysfunction in the absence of Purkinje cells should be less severe than that occurring when these cells are bursting. Given the one-to-one relationship between the firing of Purkinje cells and eye movements, this additionally indicates that erratic burst firing at least in this circuitry results in the production of involuntary movements.

3. A GAIN IN CEREBELLAR MALFUNCTION: DYSTONIA

We propose that high frequency burst firing of Purkinje cells underlies the cerebellar-induced co-contraction of agonist and antagonist muscles. While the preceding explanation could account for the production of involuntary muscle contractions, it does not indicate why burst firing of Purkinje cells would lead to dystonia, defined as the pathological co-contraction of agonist and antagonist muscle pairs (Lanska, 2010).

The EMG signal recorded during the dystonic phase of *tottering* attacks is characterized by the simultaneous discharge of reciprocal muscles (Scholle et al., 2010). This indicates that the contraction of agonist and antagonist muscle pairs overlap to a certain extent. Under normal conditions, the contraction of reciprocal limb muscles would alternate such that when the agonist contracts, the antagonist would relax and *vice versa* (Hallett et al., 1975b; Berardelli et al., 1996). How does burst firing of Purkinje cells relate to this muscle activity pattern?

When the activity of single Purkinje cells is examined during limb movements involving the flexion and extension at a single joint, concomitant EMG recordings indicate that the firing rate of Purkinje cells change in one of two ways. The firing rate of one set of Purkinje cells increases with flexion (or extension), while that of a different set of Purkinje cells decreases (Thach, 1970a; Mano and Yamamoto, 1980; Frysinger et al., 1984). In other words, a simple movement requiring the reciprocal contraction of antagonist muscles is characterized by a reciprocal change in the firing rate of two sets of Purkinje cells. This provides a mechanism that would allow Purkinje cells to ensure that each muscle is activated at the right time such that antagonist muscles activate alternately (without significant overlap in their contraction). Thus, as detailed in the introduction, by integrating sensory and cortical inputs and providing output to motor control areas, Purkinje cells can act as a translator, signaling when the contraction of a muscle should be augmented or relaxed.

One mechanism that would allow them to do so is presented in Appendix I. Purkinje cells receive excitatory mossy fiber input via granule cells that are positioned directly underneath (Bower and Woolston, 1983). In addition, the same mossy fiber input is relayed to Purkinje cells positioned laterally via a disynaptic granule cell - molecular layer interneuron – lateral Purkinje cell connection (Gao et al., 2006; Dizon and Khodakhah, 2011). The study in Appendix I tested the hypothesis that this inhibitory pathway could allow reciprocal Purkinje cell signals similar to ones observed during movement to be generated. Thus, the response of Purkinje cells either lateral or immediately above a stimulated patch of granule cells were recorded *in vitro*. It was found that the change in the firing rate of the former set of Purkinje cells was a linear

function of the strength of stimulation and reciprocal to the change occurring in the latter set. The linearity of the input-output relationship was similarly confirmed for decreases in Purkinje cell firing rate in response to stimulation of a lateral patch of granule cells *in vivo*. This indicated that the granule cell - molecular layer interneuron – Purkinje cell pathway could instigate reciprocal activity of Purkinje cells similar to the ones observed *in vivo* in association with the contraction of reciprocal muscles. Within this framework, it is easy to understand how modulation of a Purkinje cell's firing by intrinsic, pathological factors could lead to co-contraction of agonist and antagonist muscles.

Given that the peak of the attacks are characterized by long periods of bursts firing by virtually all Purkinje cells that were examined, and given the large number of Purkinje cells in the cerebellar cortex, it is reasonable to expect that the bursts of activity of some Purkinje cells will overlap to some degree. To be clear, this mechanism would not require the bursts of activity in a large number of Purkinje cells to overlap completely. As described, the pattern of muscle activity during the peak of *tottering*'s attacks is such that the bursts of discharge of VL and BF muscles are *partially* overlapped with interposed sequences of short bursts some of the time (Scholle et al., 2010). Such overlapping bursting activity in reciprocal sets of Purkinje cells could ultimately signal reciprocal sets of muscles to contract simultaneously and give rise to the EMG patterns observed in the *tottering*. This is but one mechanism that would explain our observations. As mentioned, the relationship between Purkinje cell firing and the execution of complex movements is not entirely clear. An experiment similar to the one performed in the preceding chapter but combined with EMG monitoring of the activation of limb muscles could further test our hypothesis. Thus, one would predict that when the *tottering* walked

on a treadmill in the absence of an attack, the firing rate of a given set of Purkinje cells would increase and decrease in phase with the contraction of a given muscle and out of phase with a reciprocal set of Purkinje cells. Once two such reciprocal Purkinje cells are located, an attack could be triggered and the activity of these cells monitored throughout the different phases of the attack. Based on our hypothesis, we would predict that during the peak of an attack, the co-contraction of the agonist and antagonist muscle pairs associated with this set of Purkinje cells would correlate with burst firing in both cells. On the other hand, our hypothesis would be disproved if during an attack, the bursting activity of two sets of Purkinje cells whose activities are known to correlate with that of a reciprocal pair of muscles did not significantly overlap during the co-contraction of the same muscle pair.

D. ERRATIC CEREBELLAR OUTPUT AS COMMON MECHANISM

There are numerous ways by which the final output from the cerebellum can be altered. The activity of DCN neurons is the end result of their integration of inhibitory input from Purkinje cells as well as excitatory ones from mossy and climbing fiber collaterals (Matsushita and Iwahori, 1971a; Chan-Palay, 1977a). We would predict that altering any of these components will diminish either the information content or the signal-to-noise ratio, or change the information contained within the output signal altogether.

1. PATHOPHYSIOLOGY OF PURKINJE CELLS IN CEREBELLAR DISEASE

Purkinje cells process a vast amount of excitatory and inhibitory inputs, which modulate their extremely regular intrinsic pacemaking. In Chapter 4, it was found that the *klhl1* deletion alters the excitability of Purkinje cells by causing a decrease in the size of

their dendrites, which translates into a higher input resistance and a smaller membrane capacitance. The studies in Chapters 2 and 3 implicate an entirely different mechanism by which the combination of a diminished calcium current density and a stress-induced increase in norepinephrine release, presumably by altering SK channel function, change the properties of Purkinje cells. The outcome of the *tottering* mutation and the *klhl1* deletion ultimately propagate to output neurons and manifest as erratic firing. Although the molecular underpinnings of the *car8* mutation have not been explored, in this mouse these translate into an altered DCN activity as well.

Our results extend the growing evidence implicating changes in the excitability of Purkinje cells in the pathophysiology of inherited ataxia. Purkinje cells were similarly found to be more excitable in mice expressing the full human disease gene underlying SCA3 (Shakkottai et al., 2011). This was attributed to a net increase in a depolarizing current which arose from an increased inactivation of the Kv3.1 channel (Shakkottai et al., 2011). The reduction in the current contributed by these channels to the repolarization phase of the action potential drove Purkinje cells into depolarization block and effectively silenced the activity of a majority of them. In this mouse there was strong evidence that the altered excitability of Purkinje cells was culpable for the motor deficits since the firing rate regularized and motor symptoms improved upon administration of a K_{Ca} channel activator (Shakkottai et al., 2011). Similarly in EA1 the mutated gene codes for Kv1.1 channels and markedly alters the channel's properties so as to cause a slower inactivation and a faster recovery from inactivation (Maylie et al., 2002). Interestingly the EA1 mouse model shows an increased inhibition and excitability (Zuberi et al., 1999; Herson et al., 2003) as well as erratic firing of Purkinje cells (Davies, 2007). SCA13 is

another ataxia that implicates potassium channel dysfunction, specifically Kv3.3 (Waters et al., 2006). In the Kv3.3 knockout mouse, gait abnormalities occur concomitantly with a significantly altered Purkinje cell action potential waveform (Zagha et al., 2008).

Our results suggest that aberrant Purkinje cell firing can instigate hyperkinetic symptoms. A recent study linking Purkinje cell dysfunction to the expression of dyskinesia parallels our findings. In this study mice with targeted disruption of the fibroblast growth factor 14 locus (*FGF14*), mutations of which underlie SCA27, developed both ataxia as well as dyskinesia and dystonia (Wang et al., 2002). Purkinje cells in the mutant mice were unable to sustain repetitive firing and in fact 80% of cells recorded *in vitro* were found to be quiescent (Shakkottai et al., 2009). This was linked to a reduced expression of Nav1.6 channels. The crucial role these channels play in normal cerebellar function is highlighted by the fact that knocking them out in Purkinje cells impairs coordination (Levin et al., 2006).

2. OTHER SOURCES OF ABERRANT CEREBELLAR OUTPUT

While the mutation in the *tottering* results in altered channel properties, in Chapter 4 the excitability of Purkinje cells were found to be altered as a result of deleting *klhl1*, a gene whose protein product does not directly suggest the presence of ion channel abnormalities. This finding adds to the growing evidence in the literature indicating that the diseased genes in ataxias need not impair ion channel function directly in order to alter Purkinje cell excitability.

The mutant mice studied in this thesis did not exhibit extensive neuronal degeneration. However, the same mechanism could explain motor symptoms that are instigated solely by the degeneration of Purkinje cells. Given that multiple Purkinje cells

converge onto a single DCN neuron (Eccles, 1973; Person and Raman, 2012), it is thought that the latter extracts the information that is common to many Purkinje cells and disregards the noise unique to each (Eccles, 1973). Averaging the activity of many neurons will reduce noise that is private to each Purkinje cell to a degree that depends on the size of the converging Purkinje cell population (Shadlen and Newsome, 1994). The ultimate outcome of a reduction in the number of Purkinje cells would therefore be predicted to decrease the quality of the averaged DCN signal and degrade its information content. This hypothesis could be tested by examining the activity of DCN neurons in response to the progressive degeneration of Purkinje cells. For example in the purkinje cell degeneration mutant, Purkinje cells gradually die starting at postnatal week 3 such that virtually none survive by the time the mouse is 5 weeks old (Mullen et al., 1976). We would predict that if the activity of DCN neurons were recorded at different time points between 3 and 5 weeks, they would become progressively more erratic.

Additionally alterations in the intrinsic properties of DCN neurons, or their synaptic inputs, might be a source of information loss or introduction of additional noise with the same outcome that the signal-to-noise ratio of the information encoded in their activity is reduced. For example silencing SK channel in the DCN using a dominant-negative construct results in ataxia and erratic firing of nuclear neurons (Shakkottai et al., 2004).

3. Stress as a modifier of Purkinje cell activity

In Chapter 2, a mechanism for the induction of motor symptoms in the *tottering* mouse was proposed and investigated. Although there exists multiple triggers of attacks, such as caffeine and ethanol, stress was chosen due to the fact that it is shared among

virtually all episodic channelopathies (Jen, 2000; Finsterer, 2008; Hansen et al., 2011; Lee et al., 2012). How applicable would the proposed mechanism be to other triggers? The answer to this question remains to be determined. There are as yet very few promising leads with regard to the physiological mechanism underlying the triggers' action. In a recent study, the mechanism of action of caffeine was examined in a transgenic mouse model of paroxysmal nonkinesinegic dyskinesia (PNKD), which expresses attacks of motor dysfunction (Lee et al., 2012). It was found that systemic injections of the A_{2A} adenosine receptor antagonist triggered attacks of dyskinesia (Lee et al., 2012). In our study, the induction of attacks in *tottering* mice was investigated using stress as a trigger and implicated an increased release of norepinephrine onto the cerebellum. Given that caffeine is capable of potentiating the release of norepinephrine via A_{2A} receptors (Barraco et al., 1995; Moreau and Huber, 1999; Lorbar et al., 2004), examining whether the proposed mechanism involves an increased release of norepinephrine, would help answer the interdependence of these two mechanisms.

There exists evidence suggesting that both caffeine and ethanol are capable of increasing norepinephrine levels in the cerebellum. At physiological concentrations, caffeine is an antagonist of presynaptic adenosine receptors (Barraco et al., 1995). It is known that through its effect on adenosine receptors, caffeine potentiates the release of several neurotransmitters, including that of norepinephrine (Berkowitz et al., 1970). In fact the caffeine-induced increase of norepinephrine release is inhibited by clonidine, the same α_2 agonist shown to prevent attacks in the *tottering* in Chapter 3 (Grant and Redmond, 1982; Galloway and Roth, 1983). Similarly, ethanol inhibits the reuptake of norepinephrine from the extracellular space (Israel et al., 1973; Lin et al., 1993),

including in the cerebellum (Lin et al., 1997). Collectively these studies provide sufficient evidence to warrant further investigation of norepinephrine's role in the induction of attacks by caffeine and ethanol. For example, measuring norepinephrine levels in the *tottering* cerebellum in response to caffeine and ethanol triggered-attacks could help further test the proposed hypothesis.

4. Propagation of Purkinje cell malfunction to DCN

In all three mutant mice, it was found that the altered behavior of Purkinje cells was accompanied by a change in the pattern of DCN activity. This is an important finding because the outcome of altered Purkinje cell behavior on cerebellar output neurons, to our knowledge, has never been studied. For any given mutation-induced change in the cerebellar cortex, it is entirely possible that compensatory mechanisms at the level of the Purkinje neuron – DCN synapse resolve the erratic cerebellar cortical output. Moreover, given the convergence of multiple Purkinje cells onto a single DCN neuron (Eccles et al., 1967; Person and Raman, 2012), moderate noise in the output from the cerebellar cortex could be averaged out (Eccles, 1973). In fact using this same reasoning the finding that changes in the precision of Purkinje cell pacemaking could underlie ataxia in mutant mice was challenged (Strupp et al., 2008; Alvina and Khodakhah, 2010b). The effect of altering the regularity of Purkinje cells on their target neurons was examined in a computational study and it was concluded that such changes would not alter the behavior of nuclear cells unless the firing of Purkinje cells was highly synchronous (to the point of physiological irrelevance) (Strupp et al., 2008). Our results disagree with this study given that in the mutant mice studied here, there was strong evidence indicating that

malfunction occurred in the cerebellar cortex and changes in the DCN firing pattern were reliably identified.

It must be noted that our results do not provide the evidence necessary to unarguably claim that it is the altered firing of Purkinje cells that drives the erratic DCN firing. Even though a more regular Purkinje cell firing pattern accompanies the improvement of motor symptoms with K_{Ca} channel activators, that it is chlorzoxazone's effect on Purkinje cells that alleviates the symptoms cannot be assumed. Moreover, whether activators act on Purkinje cell or on DCN neurons remains a mystery. Given the similar players involved in the regulation of the excitability of both Purkinje cells and DCN neurons, the role of nuclear neurons in the pathogenic mechanism must be considered such that careful therapeutic strategies can be implemented.

The output from the cerebellum is a result of the nuclear neurons' integration of input from climbing and mossy fibers which relay signals originating in myriad locations, as well as the output from the cerebellar cortex (Zheng and Raman, 2010; Haines and Dietrichs, 2012). Collectively the evidence from this thesis indicates that a change in any of the signals converging on DCN neurons could alter cerebellar output such that the signal it conveys is either less accurate or erroneous, resulting in the expression of motor symptoms that lie somewhere along the spectrum delimited on one end by ataxia and on the other by dystonia.

The finding that dystonia is associated with high frequency burst firing of DCN neurons extends those from several prior studies which tested the hypothesis that an "overly active" cerebellum results in dystonia. Thus, the selective lesioning of the DCN

was reported to entirely abolish the symptoms of the dystonic rat (LeDoux and Lorden, 1998). In fact following lesion of the nuclei, the dystonic rat, which dies prematurely due to a worsening of its symptoms, was able to survive as long as wild types. Subsequent experiments in the *tottering* (Neychev et al., 2008) as well as the *lethargic* mouse model of paroxysmal dyskinesia (Devanagondi et al., 2007) also showed that removing the cerebellum abolished the dystonic symptoms. Similarly, kainic acid injections into the cerebellum cause dystonia in otherwise normal mice (Pizoli et al., 2002) and in a recent experiment, the selective activation of AMPA receptors was shown to induce the same symptoms (Fan et al., 2012). Interestingly, the outcome of at least one human study parallels our findings as well. In order to investigate the common association of involuntary jerky limb movements (myoclonus attacks) with cerebellar tumors in humans, depth electrode recordings were performed in a patient prior to tumor resection (Koh et al., 2010). The activity of DCN neurons were highly erratic and in fact the involuntary muscle contractions disappeared upon tumor resection, leading the authors to conclude that the involuntary movements were likely caused by paroxysmal abnormal output from the cerebellum (Koh et al., 2010).

Based on the collective findings of these studies and ours, it can be inferred that unlike ataxia, erroneous signals mediating dyskinesia and dystonia are likely generated by the cerebellum. In other words, while cerebellar ataxia results from a loss of cerebellar function, as indicated by the fact that cerebellar lesions give rise to it in humans (Holmes, 1917), dyskinesia and dystonia result from a change in cerebellar output. This would provide a unifying physiological basis for the seemingly contradictory observations that ataxia decreased cerebellar function causes ataxia and alleviates dystonia.

5. PROPAGATION OF DCN MALFUNCTION BEYOND THE CEREBELLUM

The proposed mechanism is based on the assumption that the aberrant cerebellar output patterns alter neuronal activity at downstream motor regions. Given the extensive projections of cerebellar output neurons to cortical and brainstem motor systems detailed throughout the thesis, there are numerous pathways through which such dramatic changes in cerebellar output can alter muscular activity.

For example, it is known that stimulation of the cerebellar dentate nucleus modulates the firing pattern of both basal ganglia (Li and Parker, 1969; Ratcheson and Li, 1969) and red nuclei neurons (Massion, 1961; Tsukahara et al., 1967). In fact, the involvement of the basal ganglia in the expression of *tottering*'s paroxysmal dystonia was recently examined. In this study, the *tottering* attacks were found to associate with a significant decrease in striatal dopamine overflow as measured by microdialysis (Neychev et al., 2008). Interestingly, striatal lesions that did not cause overt motor symptoms on their own increased the duration of *tottering*'s attacks, providing further evidence that these two regions may interact in the expression of symptoms (Neychev et al., 2008). This suggests that the altered cerebellar output in the *tottering* mouse may have functional implications in the activity of striatal neurons.

Exactly how abnormal bursting activity of nuclear neurons alters the firing properties of premotor neurons in the basal ganglia, thalamus, brainstem motor systems and/or the cerebello-spinal tract remains to be determined and is an active topic of research in our laboratory.

E. IMPLICATIONS FOR ETIOLOGY AND TREATMENT

1. POTENTIAL IMPLICATIONS FOR DEGENERATIVE DISORDERS OF THE CEREBELLUM

Current treatments for ataxia generally involve rehabilitative therapies to improve motor function or delay its deterioration combined with additional drug therapies targeting the specific non-ataxic symptoms of the patient (Marmolino and Manto, 2010; Klockgether and Paulson, 2011). Clinical trials testing the efficacy of drugs traditionally thought to provide some relief for ataxic symptoms are in general sparse due to the small patient population and heterogeneous etiology (Marmolino and Manto, 2010). Combined with the results in Chapters 4 and 5 and the studies described here, activators of K_{Ca} channels are likely to be a viable option for the treatment of cerebellar disease in humans. This is not a novel suggestion by any means, our data adds to the emerging research by highlighting that this approach may provide benefits in a wide array of hereditary ataxias, and second that its applicability to syndromes in which ion channel dysfunction is not directly implicated should be considered. The data available with regard to the extent of neurodegeneration in the early stages of SCAs is scarce (but growing, for example (Seidel et al., 2012). Based on post-mortem findings, the SCA symptoms are generally assumed to result from neurodegeneration by default, when in fact in mouse models symptoms onset prior to cell death (Clark et al., 1997; He et al., 2006; Shakkottai et al., 2011). The importance of characterizing the role of dysfunction *versus* degeneration is best illustrated by the fact that a mutation that is associated with cerebellar malformations in humans can cause similar symptoms in mice without any apparent changes in brain morphology (Chapter 5). Indeed, even though the affected patient population is limited to a few families, a finding that has consistently been reported is that among siblings, the

extent of morphological abnormalities associated with the *CA8* mutation does not correlate with the severity of ataxia (Kaya et al., 2011). That the *waddles* mouse recapitulates the motor symptoms of humans with no apparent morphological abnormalities or cell loss (Jiao et al., 2005; Kaya et al., 2011) similarly suggests a role for abnormal physiology in the expression of symptoms.

At least in SCA8 and in SCA3 mice, the motor dysfunction seems to occur in the absence of neurodegeneration, which has important implications for the treatment of symptoms in SCA patients (Shakkottai et al., 2011). While there are no viable options to stop or slow the associated neuronal death yet, given that the degeneration progresses slowly in most cases, therapeutic agents that restore aberrant physiology may provide benefits to patients for a substantial amount of time. The improvement of motor deficits in the *klhl1* knockout in response to chlorzoxazone treatment further supports this possibility.

Evidence for physiological changes associated with several cerebellar inherited disorders is growing, however the progressive nature of many of these cannot be accounted for by altered physiology and incriminates degeneration rather than dysfunction. Some SCAs, especially ones associated with polyglutamine repeats, are accompanied by such extensive degeneration that targeting Purkinje cells is likely to be of no avail (Seidel et al., 2012). Recent work with the SCA1 mouse model illustrates this point very well. In this mouse it was found that early changes in Purkinje cell physiology are though to result from changes in the trafficking of potassium channels (Hourez et al., 2011). Thus, the potassium channel activator 4-AP was administered in an attempt to restore normalcy into the cell's behavior and alleviate the symptoms. Interestingly, this

strategy proved to be successful when the mice were young, but no improvement of motor symptoms occurred when 4-AP was administered to older mice (Hourez et al., 2011). It was concluded that cell loss might have been too extensive in older animals due to the progressive nature of the degeneration (Hourez et al., 2011).

As delineated above, any significant change in the input conveyed to DCN neurons can in principle alter its output. Therefore, the same pathomechanism that translates a change in cerebellar physiology into motor symptoms could in principle account for ataxias resulting strictly from neuronal cell loss. Interestingly most mutations characterized to date seem to alter the properties of Purkinje cells rather than that of DCN. Alternatively their effects on nuclear neurons may not be as well characterized. Nevertheless in the absence of compensatory mechanisms, the effect is translated into an altered output from nuclear neurons, as illustrated by the significant correlation between their aberrant firing and the severity of the motor symptoms. Whether pharmacologically decreasing nuclear neurons' presumed hyper-excitability would have therapeutic effects remains to be determined.

The finding that oral administration of K_{Ca} channel activators decreases the frequency of severe attacks in the *tottering* corroborates the conclusion that decreasing the excitability of DCN neurons to prevent them from bursting at high frequencies might be an option in the treatment of cerebellar dyskinesias and dystonias. In fact, recent clinical trials using riluzole, another potassium channel activator has shown some benefits for the treatment of EA2 symptoms (Ristori et al., 2010). The current treatment of choice for EA2 is the carbonic anhydrase inhibitor acetazolamide, whose therapeutic mode of action remains a mystery. Interestingly, acetazolamide directly activates vascular

K_{Ca} channels, making it tempting to speculate that its mode of action might be similar to that of chlorzoxazone (Pickkers et al., 2001).

2. POTENTIAL IMPLICATIONS OF A COMMON PATHOGENIC MECHANISM

Taken together, our results and those in the literature have begun to pave the way towards an understanding of the pathogenic mechanisms involved in a large group of ataxias. The importance of this is two-fold. First, similar therapeutic strategies may well prove beneficial in the seemingly heterogeneous patient population. This is illustrated by the finding that activators of K_{Ca} channels have to date been shown to improve the ataxic symptoms in mouse models of SCA3 (Shakkottai et al., 2011), EA2 (Walter et al., 2006; Alvina and Khodakhah, 2010b), *CA8* syndrome (Chapter 5) and SCA8 (Chapter 4).

Second, the existence of common physiological trends suggests that in principle the pathomechanisms that underlie the neuronal degeneration in SCA patients might be shared as well. In fact, an emerging hypothesis in the field is that a single mechanism gives rise to both the disrupted neuronal physiology and the subsequent degeneration of neurons. Thus, in this thesis it was suggested that the symptoms of cerebellar dysfunction occur due to the ultimate manifestation of various mutations as changes in the pattern of cerebellar output. Similarly, it has been argued that the heterogeneous set of mutated genes involved in inherited ataxias all converge to alter Purkinje cell calcium signaling pathways (Kasumu and Bezprozvanny, 2010). In fact it was hypothesized that the same changes in calcium signaling pathways first give rise to physiological deficits then proceed to damage and eventually kill Purkinje cells (Kasumu and Bezprozvanny, 2010). It must be noted that given our findings, some contradictions arise from the fact the hypothesis delineated above ultimately predicts that it is the block of calcium channels

that should benefit both the physiological as well as the neurotoxic effects of mutations. (Chen et al., 2008; Liu et al., 2009; Kasumu and Bezprozvanny, 2010). In fact long-term administration of dantrolene, a compound that inhibits the release of calcium from intracellular stores, was successfully used to improve motor dysfunction in mouse models of SCA2 and 3 (Chen et al., 2008; Liu et al., 2009). In contrast, we show here that increasing the affinity of K_{Ca} channels for calcium improves motor symptoms. It is impossible to compare our results with these findings given that first, in this study motor dysfunction was assessed after the long-term administration of dantrolene, and second due to the fact that motor dysfunction cannot ever be reliably assessed in the short term following administration of dantrolene due to the marked decrease in muscle tone that accompanies it (Liu et al., 2009). This contradiction is further illustrated by the fact that the extent of decrease in calcium current that results from various *CACNA1A* mutations positively correlates with the severity of the ensuing symptoms (Saito et al., 2009) such that the complete loss of P/Q- channel function in the *CACNA1A* knockout results in severe dystonia and premature death (Fletcher et al., 2001). Although seemingly dissonant, the two hypotheses are likely not contradictory given the fact that in Purkinje cells the bulk of the calcium that activates K_{Ca} channels is contributed by P/Q-type voltage-gated calcium channels rather than intracellular stores (Edgerton and Reinhart, 2003). It is beyond the scope of this thesis to further evaluate the calcium signaling hypothesis with regard to its role in neurodegeneration. However, given that two of the mutated genes studied here can directly be linked to calcium function, P/Q- channels in the *tottering* and IP_3 receptors in *waddles*, with cautious optimism one could speculate that the identification of therapeutic strategies aimed towards repairing pathophysiology

could extend to the research relating to the degenerative aspect of SCAs that renders them an incurable disorder.

F. IMPLICATIONS FOR INHERITED MOVEMENT DISORDERS

Given the concomitance of dyskinesia and dystonia with cerebellar disease, the pathophysiology associated with their occurrence was investigated. The resulting evidence indicates that the cerebellum can give rise to dyskinesia and dystonia when they occur as part of the symptomatology of an inherited ataxia. The rationale for this study was not only to better understand inherited ataxias, but to evaluate the possibility for cerebellum's involvement in the pathogenesis of inherited forms of dystonia as well. As described in detail in the introduction, symptoms involving increased muscular activity have traditionally been thought to result from dysfunction of the basal ganglia. This assumption is a result of clinicians initially describing motor disorders based on their primary features and pathological hallmarks, a remarkable feat in the absence of modern imaging tools, that ultimately led to the static definition of hyperkinetic movement symptoms as basal ganglia disorders. However, the widespread localization of lesions in secondary dystonia questions the veracity of this assumption (Jinnah and Hess, 2006; Ozelius et al., 2011; Standaert, 2011). Moreover, primary dystonias do not associate with imaging abnormalities in any specific brain region (Ozelius et al., 2011; Standaert, 2011). Given that the deleterious effects of genetic mutations, unlike lesions, tend to be global rather than focal, and that a broad spectrum of additional symptoms that accompany primary dystonia, it is likely that additional neural structures likely play a role in pathomechanism. For example, the cerebellum is implicated in inherited forms of dystonia such as DYT6, 7, 13 and 17 (Ozelius et al., 2011). Moreover, ataxia as well as

other “cerebellar symptoms” often present as part of the dystonia plus syndromes DYT 9, 10, 11, 12 (Ozelius et al., 2011). Our findings complement emerging research and clinical findings indicating the need for a paradigm shift with regard to the etiology of dystonia and the necessity to consider the cerebellum as a potential instigator of symptoms (Sadnicka et al., 2012). For example, the motor episodes observed in the PNKD transgenic mouse was attributed to dysfunction originating in the basal ganglia due to increased *c-fos* activity in this region after the induction of an attack (Lee et al., 2012). In fact, this finding is paralleled in the *tottering* mouse which also exhibits increased *c-fos* activity in the same region following the induction of an attack, as well as in the cerebellum (Campbell and Hess, 1998). However, the increased *c-fos* expression in the cerebellum temporally precedes that in the striatum. Whether any changes occurred in the cerebellum during attacks in PNKD mice was not reported and would be a welcome study. It is increasingly apparent that most, if not all, movement disorders involve problems within multiple components of the motor control circuitry, their aberrant interaction or at the very least the interaction of a dysfunctional component with additional elements of the circuitry (Niethammer et al., 2011). Examining the pathology of each component is certainly a necessary step to understand its dysfunction as a whole.

CONCLUDING REMARKS

The significance of this thesis is in the fact that based on insight from clinical and animal studies implicating the cerebellum in the expression of various symptoms; a physiological basis with regard to its involvement in different forms of inherited syndromes was dissected. In fact a gain of cerebellar function had previously been demonstrated with regard to dystonia in several mouse models based on the fact that removing the cerebellum abolished symptoms. Moreover, that ataxia results from a loss of cerebellar function (a decreased ability of Purkinje cells to encode information) was also known prior to the undertaking of these studies. However, the physiological correlates of cerebellar dyskinesia and dystonia were unknown, precluding the formulation of a unifying hypothesis that would explain their co-occurrence as part of the symptomatology of myriad inherited disorder. This has important implications for the treatment of cerebellar disorders in general and additionally highlights the need to consider cerebellar dysfunction as part of the etiology of inherited movement disorders as well. In Chapters 2-5, the firing of Purkinje and DCN neurons were found to be highly irregular in a manner that correlated with the severity of associated symptoms. Based on the overall hypothesis that aberrant cerebellar output patterns contribute to the motor symptoms in these mice, it was predicted that restoring them to normalcy would alleviate symptoms. Consistently with this hypothesis, it was found that oral administration of the K_{Ca} channel activator chlorzoxazone resulted in improved performances on the rotarod as well as decreased motor disability scores. Moreover, that the improvement of symptoms is associated with more regular firing of Purkinje cells was demonstrated *in vivo*. As a result, a mechanism implicating a stress-induced decrease in SK channel activity was

additionally investigated. It must be emphasized however that the improvement of symptoms with chlorzoxazone neither proves the action of chlorzoxazone through K_{Ca} channels nor that it is the diminished activity of K_{Ca} channels that ultimately cause irregular and burst firing of Purkinje cells.

The persistence of irregular DCN neuronal activity across seemingly unrelated genetic mutations suggests that it is a common point of convergence for the deleterious effects underlying cerebellar syndromes. Additionally, the fact that altered physiology can account for motor dysfunction when they are assumed to result from degeneration must certainly be taken into account as an alternative hypothesis for cerebellar diseases that associate with neuronal loss. DCN neurons provide the sole output from the cerebellum and their disruption unarguably results in motor deficits. It is interesting to note that while the collective findings from a multitude of studies have begun to unravel the signals DCN neurons generate in association with disease, the nature of the signals they encode to mediate healthy motor function remains to be elucidated. If the history of cerebellar research is any indication, it is likely that cerebellar function will ultimately be understood in large part through the study of its dysfunction.

REFERENCES

Allen GI, Tsukahara N (1974) Cerebrocerebellar communication systems. Physiol Rev 54:957-1006.

Alvina K, Khodakhah K (2008) Selective regulation of spontaneous activity of neurons of the deep cerebellar nuclei by N-type calcium channels in juvenile rats. The Journal of physiology 586:2523-2538.

Alvina K, Khodakhah K (2010a) The therapeutic mode of action of 4-aminopyridine in cerebellar ataxia. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7258-7268.

Alvina K, Khodakhah K (2010b) KCa channels as therapeutic targets in episodic ataxia type-2. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7249-7257.

Asanuma C, Thach WT, Jones EG (1980) Nucleus interpositus projection to spinal interneurons in monkeys. Brain research 191:245-248.

Asanuma C, Thach WT, Jones EG (1983) Brainstem and spinal projections of the deep cerebellar nuclei in the monkey, with observations on the brainstem projections of the dorsal column nuclei. Brain research 286:299-322.

Asanuma H, Fernandez J, Scheibel ME, Scheibel AB (1974) Characteristics of projections from the nucleus ventralis lateralis to the motor cortex in the cats: an anatomical and physiological study. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 20:315-330.

Baloh RW (2012) Episodic ataxias 1 and 2. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:595-602.

Baloh RW, Jen J (2000) Episodic ataxia type 2 and spinocerebellar ataxia type 6.

Barraco RA, Clough-Helfman C, Goodwin BP, Anderson GF (1995) Evidence for presynaptic adenosine A2a receptors associated with norepinephrine release and their desensitization in the rat nucleus tractus solitarius. JNeurochem 65:1604-1611.

Berardelli A, Hallett M, Rothwell JC, Agostino R, Manfredi M, Thompson PD, Marsden CD (1996) Single-joint rapid arm movements in normal subjects and in patients with motor disorders. Brain : a journal of neurology 119 (Pt 2):661-674.

Berkowitz BA, Tarver JH, Spector S (1970) Release of norepinephrine in the central nervous system by theophylline and caffeine. EurJPharmacol 10:64-71.

Blumkin L, Michelson M, Leshinsky-Silver E, Kivity S, Lev D, Lerman-Sagie T (2010) Congenital ataxia, mental retardation, and dyskinesia associated with a novel CACNA1A mutation. Journal of child neurology 25:892-897.

Bower JM, Woolston DC (1983) Congruence of spatial organization of tactile projections to granule cell and Purkinje cell layers of cerebellar hemispheres of the albino rat: vertical organization of cerebellar cortex. JNeurophysiol 49:745-766.

Bremer F, Bonnet V (1953) Action de la strychnine sur les résponses sensorielles et sur les potentiels électriques spotanés de l'écorce cérébelleuse: L'activité convulsive du cervelet. Folia psychiat, neurol et neurochir néerl 56:438-446.

Brookhart JM, Moruzzi G, Snider RS (1950) Spike discharges of single units in the cerebellar cortex. J Neurophysiol 13:465-486.

Campbell DB, Hess EJ (1998) Cerebellar circuitry is activated during convulsive episodes in the tottering (tg/tg) mutant mouse. Neuroscience 85:773-783.

Chen X, Tang TS, Tu H, Nelson O, Pook M, Hammer R, Nukina N, Bezprozvanny I (2008) Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:12713-12724.

Clark HB, Burright EN, Yunis WS, Larson S, Wilcox C, Hartman B, Matilla A, Zoghbi HY, Orr HT (1997) Purkinje cell expression of a mutant allele of SCA1 in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:7385-7395.

Cohen B, Goto K, Shanzer S, Weiss AH (1965) Eye movements induced by electric stimulation of the cerebellum in the alert cat. Experimental neurology 13:145-162.

Cuenca-Leon E, Banchs I, Serra SA, Latorre P, Fernandez-Castillo N, Corominas R, Valverde MA, Volpini V, Fernandez-Fernandez JM, Macaya A, Cormand B (2009) Late-onset episodic ataxia type 2 associated with a novel loss-of-function mutation in the CACNA1A gene. Journal of the neurological sciences 280:10-14.

Davies F, Adelman, J.P., and Maylie, J. G. (2007) Cerebellar Purkinje cells in a mouse model of episodic ataxia/myokemia type1 display reduced precision of tonic firing mediated by GABAergic input from cerebellar interneurons. . In: Society for Neuroscience, 2007 San Diego, CA.

Denier C, Ducros A, Vahedi K, Joutel A, Thierry P, Ritz A, Castelnovo G, Deonna T, Gerard P, Devoize JL, Gayou A, Perrouty B, Soisson T, Autret A, Warter JM, Vighetto A, Van Bogaert P, Alamowitch S, Roullet E, Tournier-Lasserve E (1999) High prevalence of CACNA1A truncations and broader clinical spectrum in episodic ataxia type 2. Neurology 52:1816-1821.

Devanagondi R, Egami K, LeDoux MS, Hess EJ, Jinnah HA (2007) Neuroanatomical substrates for paroxysmal dyskinesia in lethargic mice. Neurobiology of disease 27:249-257.

Dizon MJ, Khodakhah K (2011) The role of interneurons in shaping Purkinje cell responses in the cerebellar cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:10463-10473.

Eccles JC (1973) The cerebellum as a computer: patterns in space and time. The Journal of physiology 229:1-32.

Eccles JC, Ito M, Szenthagothai J (1967) The cerebellum as a neuronal machine. Heidelberg: Springer.

Edgerton JR, Reinhart PH (2003) Distinct contributions of small and large conductance Ca2+-activated K+ channels to rat Purkinje neuron function. JPhysiol 548:53-69.

Fan X, Hughes KE, Jinnah HA, Hess EJ (2012) Selective and sustained alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor activation in cerebellum induces dystonia in mice. The Journal of pharmacology and experimental therapeutics 340:733-741.

Finsterer J (2008) Primary periodic paralyses. Acta NeurolScand 117:145-158.

Fletcher CF, Tottene A, Lennon VA, Wilson SM, Dubel SJ, Paylor R, Hosford DA, Tessarollo L, McEnery MW, Pietrobon D, Copeland NG, Jenkins NA (2001) Dystonia and cerebellar atrophy in Cacna1a null mice lacking P/Q calcium channel activity. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15:1288-1290.

Flourens MJP (1824) Researches expérimentales sur le propriétés et fonctions du système nerveux dans les animaux vertébrés. Paris: Cervot.

Frysinger RC, Bourbonnais D, Kalaska JF, Smith AM (1984) Cerebellar cortical activity during antagonist cocontraction and reciprocal inhibition of forearm muscles. JNeurophysiol 51:32-49.

Fureman BE, Hess EJ (2005) Noradrenergic blockade prevents attacks in a model of episodic dysfunction caused by a channelopathy. Neurobiology of disease 20:227-232.

Fureman BE, Jinnah HA, Hess EJ (2002) Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. Pharmacology, biochemistry, and behavior 73:631-637.

Galloway MP, Roth RH (1983) Clonidine prevents methylxanthine stimulation of norepinephrine metabolism in rat brain. Journal of neurochemistry 40:246-251.

Gao B, Sekido Y, Maximov A, Saad M, Forgacs E, Latif F, Wei MH, Lerman M, Lee JH, Perez-Reyes E, Bezprozvanny I, Minna JD (2000) Functional properties of a new voltage-dependent calcium channel alpha(2)delta auxiliary subunit gene (CACNA2D2). The Journal of biological chemistry 275:12237-12242.

Gao W, Chen G, Reinert KC, Ebner TJ (2006) Cerebellar cortical molecular layer inhibition is organized in parasagittal zones. JNeurosci 26:8377-8387.

Giffin NJ, Benton S, Goadsby PJ (2002) Benign paroxysmal torticollis of infancy: four new cases and linkage to CACNA1A mutation. Developmental medicine and child neurology 44:490-493.

Grant SJ, Redmond DE, Jr. (1982) Methylxanthine activation of noradrenergic unit activity and reversal by clonidine. European journal of pharmacology 85:105-109.

Green MC, Sidman RL (1962) Tottering--a neuromusclar mutation in the mouse. And its linkage with oligosyndacylism. JHered 53:233-237.

Haines DE, Dietrichs E (2012) The cerebellum - structure and connections. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 103:3-36.

Hallett M, Shahani BT, Young RR (1975a) EMG analysis of patients with cerebellar deficits. Journal of neurology, neurosurgery, and psychiatry 38:1163-1169.

Hallett M, Shahani BT, Young RR (1975b) EMG analysis of stereotyped voluntary movements in man. Journal of neurology, neurosurgery, and psychiatry 38:1154-1162.

Hansen JM, Hauge AW, Ashina M, Olesen J (2011) Trigger factors for familial hemiplegic migraine. Cephalalgia : an international journal of headache 31:1274-1281.

He Y, Zu T, Benzow KA, Orr HT, Clark HB, Koob MD (2006) Targeted deletion of a single Sca8 ataxia locus allele in mice causes abnormal gait, progressive loss of motor coordination, and Purkinje cell dendritic deficits. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:9975-9982.

Herson PS, Virk M, Rustay NR, Bond CT, Crabbe JC, Adelman JP, Maylie J (2003) A mouse model of episodic ataxia type-1. Nature neuroscience 6:378-383.

Hoebeek FE, Stahl JS, van Alphen AM, Schonewille M, Luo C, Rutteman M, van den Maagdenberg AM, Molenaar PC, Goossens HH, Frens MA, De Zeeuw CI (2005) Increased noise level of purkinje cell activities minimizes impact of their modulation during sensorimotor control. Neuron 45:953-965.

Holmes G (1917) The symptoms of acute cerebellar injuries due to gunshot injuries. Brain : a journal of neurology 40:461-535.

Hourez R, Servais L, Orduz D, Gall D, Millard I, de Kerchove d'Exaerde A, Cheron G, Orr HT, Pandolfo M, Schiffmann SN (2011) Aminopyridines correct early dysfunction and delay neurodegeneration in a mouse model of spinocerebellar ataxia type 1. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:11795-11807.

Israel Y, Carmichael FJ, Macdonald JA (1973) Effects of ethanol on norepinephrine uptake and electrically stimulated release in brain tissue. AnnNYAcadSci 215:38-48.

Ito M (1984) The Cerebellum and Neural Control. New York: Raven.

Jen J (2000) Familial Episodic Ataxias and Related Ion Channel Disorders. Current treatment options in neurology 2:429-431.

Jen J, Kim GW, Baloh RW (2004) Clinical spectrum of episodic ataxia type 2. Neurology 62:17-22.

Jen JC, Graves TD, Hess EJ, Hanna MG, Griggs RC, Baloh RW, investigators C (2007) Primary episodic ataxias: diagnosis, pathogenesis and treatment. Brain : a journal of neurology 130:2484-2493.

Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, Gu W (2005) Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. Genetics 171:1239-1246.

Jinnah HA, Hess EJ (2004) Animal Models of Movement Disorders. San Diego: Elsevier.

Jinnah HA, Hess EJ (2005) The assesment of movement disorders in rodents. In: Animal Models of Movement Disorders (LeDoux MS, ed): Elsevier.

Jinnah HA, Hess EJ (2006) A new twist on the anatomy of dystonia: the basal ganglia and the cerebellum? Neurology 67:1740-1741.

Jinnah HA, Hess EJ (2008) Experimental therapeutics for dystonia. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics 5:198-209.

Jinnah HA, Hess EJ, Ledoux MS, Sharma N, Baxter MG, Delong MR (2005) Rodent models for dystonia research: characteristics, evaluation, and utility. Movement disorders : official journal of the Movement Disorder Society 20:283-292.

Kasumu A, Bezprozvanny I (2010) Deranged Calcium Signaling in Purkinje Cells and Pathogenesis in Spinocerebellar Ataxia 2 (SCA2) and Other Ataxias. Cerebellum.

Kato K (1990) Sequence of a novel carbonic anhydrase-related polypeptide and its exclusive presence in Purkinje cells. FEBS letters 271:137-140.

Kaya N, Aldhalaan H, Al-Younes B, Colak D, Shuaib T, Al-Mohaileb F, Al-Sugair A, Nester M, Al-Yamani S, Al-Bakheet A, Al-Hashmi N, Al-Sayed M, Meyer B, Jungbluth H, Al-Owain M (2011) Phenotypical spectrum of cerebellar ataxia associated with a novel mutation in the CA8 gene, encoding carbonic anhydrase (CA) VIII. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 156B:826-834.

Klockgether T, Paulson H (2011) Milestones in ataxia. Movement disorders : official journal of the Movement Disorder Society 26:1134-1141.

Koh KN, Lim BC, Hwang H, Park JD, Chae JH, Kim KJ, Hwang YS, Kim SK, Wang KC, Moon HK (2010) Cerebellum can be a possible generator of progressive myoclonus. Journal of child neurology 25:728-731.

Langer T, Fuchs AF, Chubb MC, Scudder CA, Lisberger SG (1985) Floccular efferents in the rhesus macaque as revealed by autoradiography and horseradish peroxidase. The Journal of comparative neurology 235:26-37.

Lanska DJ (2010) Chapter 33: the history of movement disorders. Handbook of clinical neurology / edited by PJ Vinken and GW Bruyn 95:501-546.

LeDoux MS, Lorden JF (1998) Abnormal cerebellar output in the genetically dystonic rat. Advances in neurology 78:63-78.

LeDoux MS, Lorden JF, Ervin JM (1993) Cerebellectomy eliminates the motor syndrome of the genetically dystonic rat. Experimental neurology 120:302-310.

Lee HY, Nakayama J, Xu Y, Fan X, Karouani M, Shen Y, Pothos EN, Hess EJ, Fu YH, Edwards RH, Ptacek LJ (2012) Dopamine dysregulation in a mouse model of paroxysmal nonkinesigenic dyskinesia. The Journal of clinical investigation 122:507-518.

Levin SI, Khaliq ZM, Aman TK, Grieco TM, Kearney JA, Raman IM, Meisler MH (2006) Impaired motor function in mice with cell-specific knockout of sodium channel Scn8a (NaV1.6) in cerebellar purkinje neurons and granule cells. Journal of neurophysiology 96:785-793.

Li CL, Parker LO (1969) Effect of dentate stimulation on neuronal activity in the globus pallidus. ExpNeurol 24:298-309.

Lin AM, Bickford PC, Palmer MR, Gerhardt GA (1993) Ethanol inhibits the uptake of exogenous norepinephrine from the extracellular space of the rat cerebellum. NeurosciLett 164:71-75.

Lin AM, Bickford PC, Palmer MR, Cline EJ, Gerhardt GA (1997) Effects of ethanol and nomifensine on NE clearance in the cerebellum of young and aged Fischer 344 rats. Brain Res 756:287-292.

Lisberger SG, Fuchs AF (1978) Role of primate flocculus during rapid behavioral modification of vestibuloocular reflex. I. Purkinje cell activity during visually guided horizontal smooth-pursuit eye movements and passive head rotation. JNeurophysiol 41:733-763.

Lisberger SG, Evinger C, Johanson GW, Fuchs AF (1981) Relationship between eye acceleration and retinal image velocity during foveal smooth pursuit in man and monkey. Journal of neurophysiology 46:229-249.

Liu J, Tang TS, Tu H, Nelson O, Herndon E, Huynh DP, Pulst SM, Bezprozvanny I (2009) Deranged calcium signaling and neurodegeneration in spinocerebellar ataxia type 2. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:9148-9162.

Lorbar M, Chung ES, Nabi A, Skalova K, Fenton RA, Dobson JG, Jr., Meyer TE (2004) Receptors subtypes involved in adenosine-mediated modulation of norepinephrine release from cardiac nerve terminals. CanJPhysiol Pharmacol 82:1026-1031.

Mano N, Yamamoto K (1980) Simple-spike activity of cerebellar Purkinje cells related to visually guided wrist tracking movement in the monkey. JNeurophysiol 43:713-728.

Mark MD, Maejima T, Kuckelsberg D, Yoo JW, Hyde RA, Shah V, Gutierrez D, Moreno RL, Kruse W, Noebels JL, Herlitze S (2011) Delayed postnatal loss of P/Q-type calcium channels recapitulates the absence epilepsy, dyskinesia, and ataxia phenotypes of genomic Cacna1a mutations. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4311-4326.

Marmolino D, Manto M (2010) Past, present and future therapeutics for cerebellar ataxias. Current neuropharmacology 8:41-61.

Massion J (1961) Contribution à l'étude de la régulation cérébelleuse du système extrapyramidal:

contrôle réflexe et tonique de la voie rubrospinale par le cervelet. Bruxelles: Editions Arscia.

Maylie B, Bissonnette E, Virk M, Adelman JP, Maylie JG (2002) Episodic ataxia type 1 mutations in the human Kv1.1 potassium channel alter hKvbeta 1-induced N-type inactivation. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4786-4793.

McMasters RE, Weiss AH, Carpenter MB (1966) Vestibular projections to the nuclei of the extraocular muscles. Degeneration resulting from discrete partial lesions of the vestibular nuclei in the monkey. The American journal of anatomy 118:163-194.

Medina JF, Lisberger SG (2007) Variation, signal, and noise in cerebellar sensory-motor processing for smooth-pursuit eye movements. JNeurosci 27:6832-6842.

Moreau JL, Huber G (1999) Central adenosine A(2A) receptors: an overview. Brain research Brain research reviews 31:65-82.

Moseley ML, Zu T, Ikeda Y, Gao W, Mosemiller AK, Daughters RS, Chen G, Weatherspoon MR, Clark HB, Ebner TJ, Day JW, Ranum LP (2006) Bidirectional expression of CUG and CAG expansion transcripts and intranuclear polyglutamine inclusions in spinocerebellar ataxia type 8. Nature genetics 38:758-769.

Mullen RJ, Eicher EM, Sidman RL (1976) Purkinje cell degeneration, a new neurological mutation in the mouse. ProcNatlAcadSciUSA 73:208-212.

Najmabadi H et al. (2011) Deep sequencing reveals 50 novel genes for recessive cognitive disorders. Nature 478:57-63.

Nashold BS, Jr., Slaughter DG (1969) Effects of stimulating or destroying the deep cerebellar regions in man. Journal of neurosurgery 31:172-186.

Neychev VK, Fan X, Mitev VI, Hess EJ, Jinnah HA (2008) The basal ganglia and cerebellum interact in the expression of dystonic movement. Brain : a journal of neurology 131:2499-2509.

Niethammer M, Carbon M, Argyelan M, Eidelberg D (2011) Hereditary dystonia as a neurodevelopmental circuit disorder: Evidence from neuroimaging. Neurobiology of disease 42:202-209.

Ozelius LJ, Lubarr N, Bressman SB (2011) Milestones in dystonia. Movement disorders : official journal of the Movement Disorder Society 26:1106-1126.

Person AL, Raman IM (2012) Purkinje neuron synchrony elicits time-locked spiking in the cerebellar nuclei. Nature 481:502-505.

Pickkers P, Hughes AD, Russel FG, Thien T, Smits P (2001) In vivo evidence for K(Ca) channel opening properties of acetazolamide in the human vasculature. BrJPharmacol 132:443-450.

Pizoli CE, Jinnah HA, Billingsley ML, Hess EJ (2002) Abnormal cerebellar signaling induces dystonia in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7825-7833.

Preston DC, Shapiro BE (2002) Needle electromyography. Fundamentals, normal and abnormal patterns. Neurologic clinics 20:361-396, vi.

Rajakulendran S, Schorge S, Kullmann DM, Hanna MG (2010) Dysfunction of the Ca(V)2.1 calcium channel in cerebellar ataxias. F1000 biology reports 2.

Raman IM, Bean BP (1999) Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. JNeurosci 19:1663-1674.

Raman IM, Gustafson AE, Padgett D (2000) Ionic currents and spontaneous firing in neurons isolated from the cerebellar nuclei. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:9004-9016.

Ratcheson RA, Li CL (1969) Effect of dentate stimulation on neuronal activity in the caudate nucleus. ExpNeurol 25:268-281.

Ristori G, Romano S, Visconti A, Cannoni S, Spadaro M, Frontali M, Pontieri FE, Vanacore N, Salvetti M (2010) Riluzole in cerebellar ataxia: a randomized, double-blind, placebo-controlled pilot trial. Neurology 74:839-845.

Roubertie A, Echenne B, Leydet J, Soete S, Krams B, Rivier F, Riant F, Tournier-Lasserve E (2008) Benign paroxysmal tonic upgaze, benign paroxysmal torticollis, episodic ataxia and CACNA1A mutation in a family. Journal of neurology 255:1600-1602.

Sadnicka A, Hoffland BS, Bhatia KP, van de Warrenburg BP, Edwards MJ (2012) The cerebellum in dystonia - help or hindrance? Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology 123:65-70.

Saito H, Okada M, Miki T, Wakamori M, Futatsugi A, Mori Y, Mikoshiba K, Suzuki N (2009) Knockdown of Cav2.1 calcium channels is sufficient to induce neurological disorders observed in natural occurring Cacna1a mutants in mice. Biochemical and biophysical research communications 390:1029-1033.

Scholle HC, Jinnah HA, Arnold D, Biedermann FH, Faenger B, Grassme R, Hess EJ, Schumann NP (2010) Kinematic and electromyographic tools for characterizing movement disorders in mice. Movement disorders : official journal of the Movement Disorder Society 25:265-274.

Seidel K, Siswanto S, Brunt ER, den Dunnen W, Korf HW, Rub U (2012) Brain pathology of spinocerebellar ataxias. Acta neuropathologica 124:1-21.

Shadlen MN, Newsome WT (1994) Noise, neural codes and cortical organization. Current opinion in neurobiology 4:569-579.

Shakkottai VG, do Carmo Costa M, Dell'Orco JM, Sankaranarayanan A, Wulff H, Paulson HL (2011) Early changes in cerebellar physiology accompany motor dysfunction in the polyglutamine disease spinocerebellar ataxia type 3. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:13002-13014.

Shakkottai VG, Xiao M, Xu L, Wong M, Nerbonne JM, Ornitz DM, Yamada KA (2009) FGF14 regulates the intrinsic excitability of cerebellar Purkinje neurons. Neurobiology of disease 33:81-88.

Shakkottai VG, Chou CH, Oddo S, Sailer CA, Knaus HG, Gutman GA, Barish ME, LaFerla FM, Chandy KG (2004) Enhanced neuronal excitability in the absence of neurodegeneration induces cerebellar ataxia. The Journal of clinical investigation 113:582-590.

Shidara M, Kawano K, Gomi H, Kawato M (1993) Inverse-dynamics model eye movement control by Purkinje cells in the cerebellum. Nature 365:50-52.

Shirley TL, Rao LM, Hess EJ, Jinnah HA (2008) Paroxysmal dyskinesias in mice. Movement disorders : official journal of the Movement Disorder Society 23:259-264.

Spacey SD, Materek LA, Szczygielski BI, Bird TD (2005) Two novel CACNA1A gene mutations associated with episodic ataxia type 2 and interictal dystonia. Archives of neurology 62:314-316.

Standaert DG (2011) Update on the pathology of dystonia. Neurobiology of disease 42:148-151.

Stein RB, Gossen ER, Jones KE (2005) Neuronal variability: noise or part of the signal? Nature reviews Neuroscience 6:389-397.

Strupp M, Kalla R, Glasauer S, Wagner J, Hufner K, Jahn K, Brandt T (2008) Aminopyridines for the treatment of cerebellar and ocular motor disorders. ProgBrain Res 171:535-541.

Strupp M, Kalla R, Claassen J, Adrion C, Mansmann U, Klopstock T, Freilinger T, Neugebauer H, Spiegel R, Dichgans M, Lehmann-Horn F, Jurkat-Rott K, Brandt T, Jen JC, Jahn K (2011) A randomized trial of 4-aminopyridine in EA2 and related familial episodic ataxias. Neurology 77:269-275.

Thach WT (1970a) Discharge of cerebellar neurons related to two maintained postures and two prompt movements. II. Purkinje cell output and input. JNeurophysiol 33:537-547.

Thach WT (1970b) Discharge of cerebellar neurons related to two maintained postures and two prompt movements. I. Nuclear cell output. Journal of neurophysiology 33:527-536.

Thach WT, Jones EG (1979) The cerebellar dentatothalamic connection: terminal field, lamellae, rods and somatotopy. Brain Res 169:168-172.

Tsukahara N, Toyama K, Kosaka K (1967) Electrical activity of red nucleus neurones investigated with intracellular microelectrodes. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 4:18-33.

Turkmen S, Guo G, Garshasbi M, Hoffmann K, Alshalah AJ, Mischung C, Kuss A, Humphrey N, Mundlos S, Robinson PN (2009) CA8 mutations cause a novel syndrome characterized by ataxia and mild mental retardation with predisposition to quadrupedal gait. PLoS genetics 5:e1000487.

Wakamori M, Yamazaki K, Matsunodaira H, Teramoto T, Tanaka I, Niidome T, Sawada K, Nishizawa Y, Sekiguchi N, Mori E, Mori Y, Imoto K (1998) Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. The Journal of biological chemistry 273:34857-34867.

Walter JT, Khodakhah K (2006) The linear computational algorithm of cerebellar Purkinje cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:12861-12872.

Walter JT, Alvina K, Womack MD, Chevez C, Khodakhah K (2006) Decreases in the precision of Purkinje cell pacemaking cause cerebellar dysfunction and ataxia. Nature neuroscience 9:389-397.

Wang Q, Bardgett ME, Wong M, Wozniak DF, Lou J, McNeil BD, Chen C, Nardi A, Reid DC, Yamada K, Ornitz DM (2002) Ataxia and paroxysmal dyskinesia in mice lacking axonally transported FGF14. Neuron 35:25-38.

Waters MF, Minassian NA, Stevanin G, Figueroa KP, Bannister JP, Nolte D, Mock AF, Evidente VG, Fee DB, Muller U, Durr A, Brice A, Papazian DM, Pulst SM (2006) Mutations in voltage-gated potassium channel KCNC3 cause degenerative and developmental central nervous system phenotypes. Nature genetics 38:447-451.

Weisz CJ, Raike RS, Soria-Jasso LE, Hess EJ (2005) Potassium channel blockers inhibit the triggers of attacks in the calcium channel mouse mutant tottering. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:4141-4145.

Yoshida T, Katoh A, Ohtsuki G, Mishina M, Hirano T (2004) Oscillating Purkinje neuron activity causing involuntary eye movement in a mutant mouse deficient in the glutamate receptor delta2 subunit. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2440-2448.

Zagha E, Lang EJ, Rudy B (2008) Kv3.3 channels at the Purkinje cell soma are necessary for generation of the classical complex spike waveform. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:1291-1300.

Zheng N, Raman IM (2010) Synaptic inhibition, excitation, and plasticity in neurons of the cerebellar nuclei. Cerebellum 9:56-66.

Zuberi SM, Eunson LH, Spauschus A, De Silva R, Tolmie J, Wood NW, McWilliam RC, Stephenson JB, Kullmann DM, Hanna MG (1999) A novel mutation in the human

voltage-gated potassium channel gene (Kv1.1) associates with episodic ataxia type 1 and sometimes with partial epilepsy. Brain : a journal of neurology 122 (Pt 5):817-825.

APPENDIX I:

EFFICIENT GENERATION OF RECIPROCAL SIGNALS BY INHIBITION[1]

Sung Min Park, Esra Tara[2] and Kamran Khodakhah

[1] This study was published in *The Journal of Neurophysiology*. 2012 May; 107(9):2453-62

[2] Esra Tara contributed to all the *in vivo* experiments.

ABSTRACT

Reciprocal activity between populations of neurons has been widely observed in the brain and is essential for neuronal computation. The different mechanisms by which reciprocal neuronal activity is generated remain to be established. A common motif in neuronal circuits is the presence of afferents that provide excitation to one set of principal neurons and, *via* interneurons, inhibition to a second set of principal neurons. This circuitry can be the substrate for generation of reciprocal signals. Here we demonstrate that this equivalent circuit in the cerebellar cortex enables the reciprocal firing rates of Purkinje cells to be efficiently generated from a common set of mossy fiber inputs. The activity of a mossy fiber is relayed to Purkinje cells positioned immediately above it by excitatory granule cells. The firing rates of these Purkinje cells increase as a linear function of mossy fiber, and thus granule cell, activity. In addition to exciting Purkinje cells positioned immediately above it, the activity of a mossy fiber is relayed to laterally positioned Purkinje cells by a disynaptic granule cell→molecular layer interneuron pathway. Here we show in acutely prepared cerebellar slices that the input-output relationship of these laterally positioned Purkinje cells is linear and reciprocal to the first set. A similar linear input-output relationship between decreases in Purkinje cell firing and the strength of stimulation of laterally positioned granule cells was also observed *in vivo*. Use of interneurons to generate reciprocal firing rates may be a common mechanism by which the brain generates reciprocal signals.

INTRODUCTION

Reciprocal activity between populations of neurons has been commonly observed in neuronal circuitry. In neural regions where sensory information is encoded, the reciprocal activity of different sets of neurons has been postulated to be a basis behind contrast enhancement. Visual, auditory, chemical, and tactile distinguishability has been attributed to the enhanced activity of one set of neurons relative to the suppressed activity of another set of neurons (Mastronarde, 1983; Calford and Semple, 1995; Moore and Nelson, 1998; Wilson and Mainen, 2006; Priebe and Ferster, 2008). For neural regions that coordinate motor behavior, the reciprocal activity of different populations of neurons plays roles in representing motor actions and coordinating muscle force (Allen and Tsukahara, 1974; Georgopoulos et al., 1986).

Given the ubiquity of reciprocal signals, it is essential to understand how these signals are generated. On the one hand, reciprocal activity in two populations of neurons may be the effect of two inversely correlated inputs. As one set of inputs provides increased excitation to one set of neurons, another set of inputs provides reduced excitation to a second set of neurons. Alternatively, a more efficient and precise method of generating reciprocal activity in neurons may be through lateral inhibition (Andersen et al., 1964). By scaling the strength of inhibition with excitation and targeting specific populations of neurons, lateral inhibition could in principle decrease the activity of one set of neurons in proportion to the increased activity of another set of neurons. While efficient, this method of reciprocal signal generation requires that inhibition has an equal yet opposite effect to that of excitation in spite of the many differences between how excitation and inhibition is transmitted to and integrated in neurons.

To determine whether lateral inhibition can generate reciprocal signals in populations of principal neurons, we first found a brain region that has a circuitry representative of many regions but is amenable to investigation. The cerebellar cortex has an anatomical center-excitation surround-inhibition circuitry similar to other brain regions, but one that is highly stereotypic. Mossy fibers provide sensory and cortical inputs to granule cells, which have ascending axons and parallel fibers that form direct excitatory inputs to only a restricted set of Purkinje cells in the sagittal plane. Molecular layer interneurons also receive excitation from granule cells and inhibit Purkinje cells lateral to the activated population of Purkinje cells (Gao et al., 2006; Dizon and Khodakhah, 2011) (Figure 1A). Thus a common set of granule cells could in principle generate reciprocal simple spike activity in two populations of Purkinje cells. Indeed, recordings from awake behaving animals routinely demonstrate populations of Purkinje cells with reciprocal simple spike firing rates, each of which encode movement related variables (Lisberger and Fuchs, 1978; Mano and Yamamoto, 1980; Frysinger et al., 1984; Medina and Lisberger, 2008).

Here we show that the strength of a single set of granule cell inputs is reciprocally encoded in the complementary firing rates of two groups of Purkinje cells. This efficient method of signal generation is made possible by individual components that linearly propagate activity within the granule cell → molecular layer interneuron→ Purkinje cell disynaptic pathway.

METHODS

All experiments were performed in accordance with the guidelines set by the Albert Einstein College of Medicine.

Cerebellar slice preparation: Wistar rats aged P13 – P22 were decapitated under halothane anesthesia. Despite anatomical and synaptic development (Pouzat and Hestrin, 1997; McKay and Turner, 2005) and granule cell migration (Fujita, 1967) throughout this age range, no differences were observed in the experimental results. Brains were quickly removed and placed in ice-cold artificial cerebro-spinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, and 11 glucose (pH 7.4 with 95% O_2, 5% CO_2). 300 μm sagittal slices of the cerebellar vermis were made using a modified Oxford vibratome. Slices were in aCSF warmed to 35°C for 1 hour and then kept for up to 4 hours at room temperature until use.

In vitro electrophysiological recordings: Slices were placed in a chamber perfused with aCSF at a rate of ~1.5 ml/min; aCSF was warmed to 35 ± 1°C and bubbled with 95% O_2, 5% CO_2. Previous work has shown that these conditions are equivalent to experiments with a flow rate of 5 ml/min (Walter and Khodakhah, 2006). For photolysis experiments, aCSF was pre-equilibrated with ~250 μM MNI-caged glutamate. Cerebellar neurons were visualized using an upright BX61WI Olympus (Tokyo, Japan) or an upright Zeiss microscope with 40×, 0.8 NA water-immersion objectives using infrared optics.

Single-unit extracellular recordings were made from cerebellar neurons from all vermal lobules using a home-made differential amplifier and glass pipettes filled with aCSF. Purkinje cells were identified by their large somata (~20 μm) and placement between the granule and molecular layers. Molecular layer interneurons were identified

by their round somata of 5 – 10 μm and placement in the molecular layer. Basket cells were defined as cells in the inner one third of the molecular layer and stellate cells in the outer two thirds. Recording electrodes were placed near the axon hillock of Purkinje cells, resulting in spike heights of ~400 μV. Recording electrodes were placed near the somata of molecular layer interneurons, resulting in spike heights of 75 – 200 μV. For dual recordings the average tip to tip distance between the two electrodes was ≈320 μm.

Whole-cell recordings were made using a Cairn Optopatch amplifier (Kent, UK). Electrodes were made from borosilicate glass and pulled to a resistance of 1 – 3 MΩ when filled with internal solution. The internal solution contained (in mM): 130 Cs-gluconate, 10 CsCl, 10 HEPES, and 3 MgATP (pH 7.2 with CsOH), and Purkinje cells were clamped at a command voltage of 0 mV. Series resistance (typically 6.5 – 8 MΩ) was compensated by 50 – 60%. For any one cell, the voltage clamp error between any set of stimulus intensities was typically << 10%. The recorded currents were blocked by bath application of $GABA_A$ and $GABA_B$ blockers picrotoxin (Sigma; St. Louis, MO) and CGP55845 (Tocris; Ellisville, MO) respectively.

Photo-stimulation with glutamate photolysis: Slices were pre-equilibrated with ~250 μM MNI-caged glutamate (Tocris), which is neither an agonist nor antagonist for glutamate receptors (Canepari et al., 2001). Ultraviolet light from a continuous multiline krypton ion laser (Innova 300C; Coherent, Santa Clara, CA) was set at 500 - 600 mW. An acousto-optical modulator (Neos) scaled the intensity (0 – 100%) and gated the light to 1 ms. The laser light was transmitted to the microscope through a fiber optic cable, collimated, and positioned *via* a pair of galvos (Cambridge Technology, Cambridge, MA). This was all driven by custom-written software. The laser light was focused to a

40 μm diameter spot on the granule cell layer. A power meter positioned at the end of the objective measured that the maximum power of this spot to be ~1.4% of the source laser light power (Walter et al., 2009). For each cell, stimulation occurred at 20 or 10 s intervals, with about 10 trials for every photolysis power. Stimulation intensities were randomly administered. In none of the experiments did the baseline firing rate of the cells change appreciably from the beginning to the end of the experiment (paired t-test: Purkinje cells: $P = 0.13$; interneurons: $P = 0.25$). Moreover, for each neuron, the averaged evoked response from the first set trials was comparable to the average response from the last set of trials (paired t-test: Purkinje cells: $P = 0.12$; interneurons: $P = 0.13$), indicating that there were no time-dependent changes in the responses of neurons. Photostimulation of a patch of granule cells results in their asynchronous activation such that the resulting EPSCs recorded in the target Purkinje cells are noisy and clearly composed of individual synaptic events, take 10-15 ms to peak, and are several fold longer in duration than EPSCs evoked by electrical stimulation of granule cells (Walter and Khodakhah, 2006, 2009). The time course of asynchronous activity of granule cells achieved with photostimulation is very comparable to that seen *in vivo* following their activation by mossy fibers with a discrete sensory input (Jaeger and Bower, 1994; Chadderton et al., 2004).

The cerebellar cortex incorporates inhibition from Golgi cells and feed-forward excitation from unipolar brush cells (UBCs). To determine whether lateral inhibition was sufficient in generating reciprocal signals, our investigation focused on inhibition from molecular layer interneurons. In our preparation photolysis of glutamate primarily activated granule cells and not UBCs because of the paucity of UBCs in our vermal slices

and also the lower excitability of the UBC relative to the granule cell (Takacs et al., 1999) (for details see (Walter et al., 2009)). The short-action of glutamate photolysis also resulted in minimal effects of Golgi-cells. Indeed, it has been previously demonstrated that under our experimental conditions selective blockade of Golgi-mediated inhibition of granule cells minimally affects the excitatory or inhibitory responses of Purkinje cells (Dizon and Khodakhah, 2011).

Both extracellular and whole-cell data were acquired at 10 kHz using an analog-to-digital converter (PCI-MIO-16XE-10; National Instruments, Austin, TX), using custom software written in LabView (National Instruments).

In vivo electrophysiological recordings: Single-unit extracellular recordings were made from Purkinje cells in 8-12 weeks old C57BL/6 under 1 – 3% isoflurane anesthesia. For these recordings, a 1.5 mm-diameter hole was drilled through the skull at -6 mm from bregma and ±1 mm from midline. Great care was taken to leave the surface of the cerebellum untouched for surface recordings. Recordings were made in lobules 4 and 5. Purkinje cells were identified by electrode depth and the presence of complex spikes and pauses. Only Purkinje cells located ~250 μm deep from the surface of the cerebellum were recorded. These arrangements allowed for accurate placement of the stimulation electrode in the granular layer and for effective blockade of $GABA_A$ receptors by topical application of 2 mM SR95531.

Individual units were acquired using a multitrode with gold cores (0.7-2 MΩ; Thomas Recording GmbH) or with platinum-quartz electrodes (2-3 MΩ; Thomas Recording GmbH) and a homemade amplifier. To target the underlying granular layer, stimulation was 100 – 200 μm deeper than the recording site, either through one of the

multitrode's sites, or using a separate tungsten wire positioned ≈350 μm lateral to the recording site in the sagittal plane. Data was filtered (80 Hz – 500 Hz high pass, 10 kHz low pass) and sampled at 20 kHz. Spikes were sorted using Offline Sorter software (Plexon Inc, Dallas, TX USA) using principle component analysis and consideration of additional parameters such as spike shape and peak and valley amplitudes and half width. Only sorted spikes that could be reasonably unambiguously identified to be from one neuron were included in the analysis.

Data analysis: The data acquisition and analysis software was written using LabView and, when needed, further analysis was performed in Matlab (Mathworks; Natick, MA) and Origin (OriginLab; Northampton, MA). Maximum and minimum instantaneous firing rates were determined as the inverse of the briefest and longest interspike intervals post-stimulus, respectively. The interspike intervals considered as part of the response were the intervals that started from the first spike before the stimulus to 1 s later, well beyond the longest conductance recordings (Figure 4E). For Purkinje cells, the baseline firing rate was calculated by averaging 24 instantaneous firing rates before the response. For interneurons, due to their slow firing rate, 15 – 24 pre-stimulus instantaneous firing rates were averaged to obtain the baseline firing rate. The change in the number of spikes was quantified as the number of spikes 500 ms before the stimulus (i.e. baseline firing rate multiplied by 500 ms) subtracted from the number of spikes during a 500 ms after the stimulus. Post-stimulus time histograms (PSTH) were made by dividing the average number of spikes per trial over a bin size of 20 ms. The duration of the response was determined from the PSTHs and was from the time of stimulus to the first instance when the firing rate returned to or below the mean baseline rate. The

number of interneuron spikes in the response was the integral of this response duration. For conductance measurements, currents were divided by the 65 mV driving force (determined by the subtracting the calculated E_{Cl} from V_{cmd}). Conductance areas were the integral from the time of stimulus to 500 ms later.

Behavioral studies: To explore the consequences of acute pharmacologic blockade of cerebellar cortical $GABA_A$ receptors on motor function behavioral experiments were performed on adult C57BL mice. Under isoflurane anesthesia four 1.5 mm wide holes were drilled in the skull at positions corresponding to paravermal and lateral lobes of the cerebellum (-6.9 mm from Bregma ±0.750 mm from midline and -6.9 mm from Bregma ±1.7 – 2 mm from midline (the average length of time taken for the first surgery was less than 5 minutes; and for the second surgery was less than 2 minutes). To expose the cerebellar cortex to drugs, the holes were filled with Gelfoam sponges (Pfizer) (Marshall and Lang, 2009) soaked in 10-25 mM SR95531 or its vehicle. Animals were sutured with the Gelfoam sponges left inside the holes, allowed to fully recover from anesthesia for at least fifteen minutes, and tested on the balance beam and observed in an open field. The average duration of time that each animal was able to maintain its balance on the beam (1 cm diameter) was determined from 10 consecutive trials. Each animal served as its own control as it received vehicle- soaked Gelfoams first, and then the vehicle-soaked Gelfoams were removed and replaced by SR95531-soaked Gelfoams. The approximate time between the application of vehicle- and SR95531-soaked Gelfoams was 1 hour. In separate experiments we found that application of SR95531 without prior exposure of the animal to vehicle produced comparable motor dysfunction, suggesting that exposure to vehicle first did not worsen the symptoms by producing

additional damage. However, we did not include these experiments in our analysis because SR95531 does not readily wash out within our experimental time frame and therefore it was not possible to use the same animal as its own control. The SR95531 solution also contained 0.01% methylene blue which allowed postmortem confirmation of the location of holes and the approximate depth of the cerebellar cortex exposed to the drugs. The dye diffused by less than ≈500 μm into the cerebellum making it extremely unlikely that any GABA receptors in the deep cerebellar nuclei were affected by the blocker.

Statistical analyses were performed using Origin software. To examine the linearity of the data, we calculated the Pearson's correlation coefficient (R). P values were acquired based on R and the degrees of freedom. To determine these parameters, raw data was used for individual cells, and mean and standard error of the mean for averaged data. Linear regressions were also determined from these data. For statistical significant differences, we used 2-sample independent and paired t-tests.

RESULTS

A common granule cell input can generate reciprocal signals

For the two reciprocal cerebellar signals to be complementary to one another, they must encode the strength of granule cell inputs with similar but mirroring functions. While other features such as excitation-induced pauses (Steuber et al., 2007) or the pattern of activity of Purkinje cells (Shin et al., 2007) have been proposed to encode cerebellar information, studies in awake behaving animals have shown only changes in the firing rate of Purkinje cells to be correlated with movement-related parameters (Thach, 1970; Lisberger and Fuchs, 1978; Mano and Yamamoto, 1980; Pasalar et al., 2006; Medina and Lisberger, 2007; Yamamoto et al., 2007; Medina and Lisberger, 2008). For direct excitation it has been demonstrated that the firing rate of Purkinje cells is a linear function of the input strength of granule cells (Walter and Khodakhah, 2006). Thus as a test of the hypothesis that a single set of granule cell inputs can generate both of the reciprocal Purkinje cell firing rates, we determined whether the input strength of granule cells is concurrently encoded in two sets of Purkinje cells as increases and decreases in firing rate (Figure 1B).

We simultaneously recorded the spontaneous firing (Llinas and Sugimori, 1980; Raman and Bean, 1999) of two Purkinje cells *in vitro* in cerebellar slices and activated a 40 μm patch of granule cells by photoreleasing glutamate. This stimulation paradigm results in the asynchronous activity of granule cells with a time course (Walter and Khodakhah, 2006; Walter et al., 2009) comparable to that seen *in vivo* following the activation by mossy fibers with a discrete sensory input (Jaeger and Bower, 1994; Chadderton et al., 2004). The size of the photolysis spot was chosen to be comparable to

the spatial extent of synapses formed by a single (or a few) mossy fiber branches. The location of the activated patch of granule cells was selected to be immediately below one of the recorded Purkinje cells and ≈200-300 μm lateral to the other (Figure 1B). Earlier studies have shown that with such an arrangement the Purkinje cells positioned above the activated patch of granule cells are excited by the granule cells whereas the ones located laterally are inhibited by disynaptic inhibition (Gao et al., 2006; Dizon and Khodakhah, 2011). The experimental conditions ensured that the concentration of photolyzed glutamate was a linear function of the photolysis power such that the input strength of the patch of granule cells was a linear function of the photolysis power (Walter and Khodakhah, 2006; Walter et al., 2009). We estimate that the range of photolysis powers used corresponds to asynchronous activation of 200-650 granule cells with each pulse (Walter and Khodakhah, 2006, 2009). As expected, asynchronous activation of a patch of granule cells promptly increased the firing of the Purkinje cell directly above the site of photolysis and decreased the firing rate of the Purkinje cell positioned laterally (Figure 1B,i). With stronger photolysis powers, greater changes in firing rate were observed in each of the two cells (Figure 1C). Since the instantaneous firing rate of Purkinje cells is a linear function of movement related variables in diverse behavioral tasks (Lisberger and Pavelko, 1986), we used this parameter to quantify the responses. For the Purkinje cell receiving direct excitation, the maximum instantaneous firing rate increased as a linear function of the strength of granule cell inputs (Walter and Khodakhah, 2006) (Figure 1D, red data points and line: $R = 0.68$, $P = 0.002$). For the Purkinje cell receiving disynaptic inhibition, the minimum instantaneous firing rate decreased as a linear function of the strength of granule inputs (Figure 1D, blue data points and line: $R = -0.41$, $P = 0.088$).

Naturally, the two reciprocal firing rates in this cell were linear functions of each other (data not shown, R = -0.89, P = 0.045). In the average of all cell pairs examined (n=5), both maximum and minimum instantaneous firing rates were each linear functions of the strength of granule cell inputs (average: Figure 1E, R = 0.99, P < 0.001; R = -0.94, P = 0.018, respectively) and to each other (average: R = -0.95, P = 0.011).

In vivo recordings show that the firing rate of Purkinje cells is modulated during behavior within a range as high as ≈250 spikes per second (sp/s) and as low as ≈5 sp/s (Lisberger and Fuchs, 1978). For increases in firing rate, the physiologically-relevant dynamic range is thus from a baseline firing of about 50 sp/s to about 250 sp/s. On the other hand, the dynamic range for decreases in activity spans from baseline to about 5 sp/s. We found that the reciprocal Purkinje cell firing rates generated by stimulation of a patch of granule cells was sufficient to smoothly alter the firing rates of the two populations of Purkinje cells to levels somewhat comparable to those observed during behavioral tasks (Thach, 1970; Lisberger and Fuchs, 1978; Frysinger et al., 1984). However, while activity of a patch of granule cells comparably changed the firing rate of the complementary Purkinje cells within their respective dynamic range, the absolute ranges were four fold different. When we examined the efficacy with which a single patch of granule cells reciprocally modulated the activity of Purkinje cells, we found the slopes of increases and decreases in activity as a function of input strength were largely reflections of each other (Figure 1D**;** the slope of each function within its respective range: excitation: 20.3 ± 5.6; inhibition: -14.1 ± 3.6). Thus our experiments show that direct excitation and disynaptic inhibition provide the requisite circuitry for the cerebellum to encode the strength of the same granule cell inputs as two sets of reciprocal

Purkinje cell firing rates. Within the context of cerebellar motor coordination, the reduced range of the reciprocal decrease in the Purkinje cell firing rate may appropriately match the asymmetric properties of muscles during relaxation compared with contraction (Todorov, 2000), although it likely that these signals are further fine-tuned by gain modifications by downstream neurons.

To scrutinize the relationship between granule cell input strength and decreases in Purkinje cell activity in more detail we recorded from 14 additional Purkinje cells while activating lateral granule cell patches throughout a larger range of photolysis powers and with a larger number of repetitions at each input strength. Within this range, the minimum post-stimulus instantaneous firing rate of the Purkinje cell shown decreased linearly as a function of the strength of granule cell inputs (Figure 2A and B, $R = -0.83$, $P < 0.0001$). Compared to its baseline spontaneous rate, inhibition transiently reduced the number of action potentials fired by the Purkinje cell after the stimulus. In the cell shown we found that the change in the number of spikes post-stimulus (for a 500 ms period) was also a linear function of the strength of granule cell inputs (Figure 2C, $R = -0.79$, $P < 0.0001$) and was directly correlated with the minimum instantaneous firing rate (Figure 2D, $R = 0.98$, $P < 0.0001$). The average minimum instantaneous firing rate and the change in the number of spikes fired post stimulus of all 14 Purkinje cells examined was linearly correlated with the input strength of granule cells (Figure 2E and F**;** $R = -0.99$, $P < 0.0001$; $R = 0.98$ $P < 0.0001$, respectively). Thus Purkinje cells linearly encode the strength of inhibitory inputs in both their minimum instantaneous firing rate and also as a reduction in the number of spikes after inhibition.

The firing rate of Purkinje cells decreases as a linear function of granule cell inputs strength *in vivo*

To determine whether the linearity of the disynaptic granule cell → molecular layer inhibition to Purkinje cells is maintained *in vivo* under background parallel fiber and interneuron activity, we monitored the discharge rate of single Purkinje cells in isofluorane anesthetized mice and, as in the experiments in brain slices, stimulated granule cells positioned laterally in the same sagittal plane. Electrical activation of granule cells resulted in immediate decreases in the firing rate of the target Purkinje cell (Figure 3A, i). Similar to that seen in cerebellar slices *in vitro*, the minimum post-stimulus instantaneous firing rate of Purkinje cells was a linear function of the stimulus intensity (Figure 3B, $R = -0.80$, $P < 0.0001$); a consistent finding in all cells examined (Figure 3C, $R = -0.98$, $P = 0.003$; n=5). No inhibitory responses were detected when $GABA_A$ receptors were pharmacologically blocked (Figure 3A, ii) (n=5) suggesting that, under our experimental conditions, fast GABAergic synaptic transmission was the sole mediator of decreases in Purkinje cell firing rate. In agreement with prior work demonstrating a critical role for cerebellar cortical $GABA_A$ receptors in generating motor coordination signals (Wisden et al., 2009), their acute pharmacologic blockade in awake behaving mice resulted in ataxia and impaired balance (average time on a balance beam reduced from 234.5 ± 38.1 s under control conditions to 26.8 ± 8.0 s when GABA receptors were blocked, $P = 0.02$, n= 4).

A series of linear input-output relationships determines the overall linearity

Although conceptually straightforward, the implementation of monosynaptic glutamatergic excitation and disynaptic GABAergic inhibition to generate reciprocal

firing rates is not trivial. In this case, to generate complementary firing rates, the input-output relationship of monosynaptic excitation and the disynaptic inhibition must both be linear. For disynaptic inhibition, this linearity may be achieved through a series of linear components, which is perhaps the simplest functional organization. To test if the disynaptic granule cell → molecular layer interneuron → Purkinje cell pathway is linear because of linear input-output components, we first examined whether interneurons linearly encode the strength of granule cell inputs in their firing rate. Consistent with earlier findings (Eccles et al., 1966), stimulation of an underlying patch of granule cells increased the firing rate of the target interneuron from its baseline spontaneous rate (Figure 4A) (the average spontaneous firing rate of basket cells was 11.9±0.1 and that of the stellate cells was 7.2±0.4 sp/s). Additionally, stronger activation of granule cells yielded greater increases in the firing rate of the interneuron (Figure 4B). Similar to that seen in Purkinje cells (Walter and Khodakhah, 2006), these increases were a linear function of the strength of granule cell inputs (Figure 4C, for cell shown in 4B, thin data points and line: $R = 0.70$, $P < 0.0001$). A linear input-output function was observed in all molecular layer interneurons examined (Figure 4C, bold data points and line: $R = 0.99$, $n = 10$; $P < 0.0001$). When basket and stellate cells were examined in isolation, the response of each class was also linear (data not shown; basket cells: $R = 0.99$, $n = 5$; $P < 0.0001$; stellate cells: $R = 0.99$, $n = 5$; $P < 0.0001$) although basket cells had a slightly steeper input-output function compared with stellate cells (ratio ≈1.3).

Considering that interneurons linearly encode the strength of granule cell inputs as increases in firing rate, we next examined whether the changes in their firing rate are also linearly transmitted to Purkinje cells as changes in GABA conductance (Figure 4D).

Photolytic activation of granule cells evoked inhibitory postsynaptic currents (IPSCs) in the target Purkinje cell, which became progressively larger and more distinguishable from background with increasing input strengths (Figure 4E). Examination of the average IPSCs revealed that the IPSCs increased with stimulus power, principally by increasing their peak amplitude and without appreciably changing kinetics (Figure 4E). The 10-90% rise time, and the decay time constant of the average evoked GABA conductances where comparable at all granule cell activation strength (10-90% rise time range 11.1±4.0 – 22.5±7.9 ms; linear correlation R=-0.19, P=0.72; decay time constant range 46.1±19 – 74.6±30.6 ms; R=0.41, P=0.43; n=4). In all 4 cells examined there was a linear relationship between the area of GABAergic conductance and the input strength of granule cells (Figure 4F, $R = 0.98$, $P < 0.0001$). In these same cells the maximum GABA conductance was also linearly correlated with the strength of granule cell input (R=0.95, P=0.004). Based on the photolysis power used to activate the patches of granule cells, we correlated the data reported in Figure 4A-C with those in Figure 4D-F. We explored whether the maximum average peak GABA conductance evoked in Purkinje cells when a patch of granule cells was activated was a linear function of the maximum instantaneous firing rate of interneurons. We found this to be the case with a linear regression line closely fitting the data (R=0.95, P=0.003). Similarly, we found that the duration of the GABA conductance evoked in Purkinje cells when the granule cells were activated was a linear function of the duration of the increase in the firing rate of interneurons ($R = 0.94$, $P = 0.006$).

The data presented so far demonstrate that interneurons linearly encode and transmit the input strength of granule cells to Purkinje cells. We next examined whether

Purkinje cell pacemaking is also linearly regulated by the strength of inhibition from molecular layer interneurons. To do so we first recorded the changes in the firing rate of a Purkinje cell in response to activation of granule cells positioned laterally to it. We then voltage-clamped the same cell and measured the GABAergic conductances produced by the same granule cell stimuli. The peri-stimulus time histogram in Figure 4H shows that the temporal changes in the firing rate of the Purkinje cell largely mirrored that of the GABA conductance. In all 5 Purkinje cells examined similarly, there was a clear inverse correlation between the minimum instantaneous firing rate and the GABAergic conductance (Figure 4I, $R = -0.95$, $P = 0.01$). This finding is consistent with previous reports of a linear relationship between the amplitude of the outward current injected into the soma of Purkinje cells and decreases in their firing rate (Mittmann and Hausser, 2007).

At least 5 interneurons mediate the observed decreases in the firing rate of Purkinje cells

We sought to estimate the number of interneurons which were activated by granule cell stimulations that drove the decreases in the firing rate of Purkinje cells reported above. With the first approach we took advantage of the finding that each individual spike fired by a single interneuron increases the Purkinje cell interspike interval by about 12% (Hausser and Clark, 1997) and linearly extrapolated the impact of multiple spikes on the increase in the interspike interval of a Purkinje cell. We found that if we assumed that five interneurons were activated by the patch of granule cells, thus inhibiting the Purkinje cell five times as much, we could reconcile the pauses produced in the activity of Purkinje cells reported here with the average number of spikes fired by

interneurons (Figure 5A, $R = 0.99$, $P < 0.001$). It should be noted that this analysis did not take into consideration the failure rate (Pouzat and Hestrin, 1997) or synaptic depression (Sakaba, 2008; Bao et al., 2010) at the molecular layer interneuron → Purkinje cell synapse and therefore the number of interneurons thus obtained is likely to be an underestimate.

We also used a second complementary approach to estimate the number of interneurons that contribute to reducing the firing rate of Purkinje cells when a patch of granule cells is activated. With this approach we compared the GABAergic conductance produced by activity of single interneurons in Purkinje cells with that obtained in our recordings. Based on the published literature, single spikes from single interneurons yield IPSCs of 80 – 330 pA (conductance peaks of 1.33 – 5.5 nS) in Purkinje cells of comparable age animals (Pouzat and Hestrin, 1997). These IPSCs decay with a single exponential decay constant of 9.3 ms (Vincent et al., 1992). Using these parameters we estimate that single spikes from an interneuron produces a GABA conductance area of 0.012– 0.050 nS*s (integrated from the peak to 500 ms later). Again assuming no synaptic failure or depression and similar to that estimated based on our first approach, comparison of our data with the published work suggests that concerted activity of ≈5 interneurons is needed to produce the responses seen in our experiments (Figure 5B).

DISCUSSION

By non-invasively monitoring the activity of Purkinje cells, here we demonstrated that a common granule cell input can generate two reciprocal firing rates in two populations of Purkinje cells. This is possible through two complementary linear input-output relationships that originate in the granule cell layer and end on Purkinje cells. Granule cells form direct excitatory inputs onto Purkinje cells, and it was previously shown that increases in the firing rate of Purkinje cells are a linear function of the input strength of these direct granule cell inputs (Walter and Khodakhah, 2006). Here we showed, both in cerebellar slices *in vitro* and in anaesthetized mice *in vivo*, that the disynaptic granule cell → molecular layer interneuron → Purkinje cell pathway also decreases the firing rate of Purkinje cells as a linear function of the input strength of granule cells. As each component of this pathway is linear, disynaptic inhibition is the equal but opposite counterpart to monosynaptic excitation. Thus in the intact animal, these two pathways should allow single mossy fiber inputs to simultaneously generate reciprocal signals. More generally, our data demonstrate the utility and efficiency of the use of lateral inhibition for generation of reciprocal signals in the brain from a common set of inputs.

Reciprocal Purkinje cells signals and the cerebellum

The anatomy of the cerebellar cortex is composed of repeated motifs that provide the requisite circuitry for reciprocal Purkinje cell signals to be generated throughout regions of the cerebellum. Indeed, functional studies demonstrate that a center-excitation surround-inhibition format exists throughout the cerebellum (Gao et al., 2006; Dizon and Khodakhah, 2011). Indeed, reciprocal firing rates of Purkinje cells are found in large

parts of the cerebellum in cats, rabbits and even primates (Eccles, 1973; Lisberger and Fuchs, 1978; Mano and Yamamoto, 1980; Chan et al., 1982; Frysinger et al., 1984; Miyashita and Nagao, 1984; Yamamoto et al., 2007; Medina and Lisberger, 2008). While the functional significance of reciprocal signals are better understood in brain regions involved in processing of sensory information, the role of these signals in the cerebellum remains to be established. As discussed below, one potential role of reciprocal signals in the cerebellum and other motor-related brain regions may be generation of the signals required for simultaneous reciprocal control of agonist and antagonist muscles.

Experimental ablation of the cerebellum in animals and examinations of patients with cerebellar lesions have collectively established that the cerebellum is involved in motor coordination (Manni and Dow, 1963; Robinson and Fuchs, 2001). It is thought that the cerebellum performs its function by generating a set of signals that, in one form or another, orchestrates the precise timing and contraction of muscles. In support of this notion, a tight correlation between the firing rate of Purkinje cells and specific motor variables has been observed for simple tasks such as the movements of the arm and eye. For movements of the arm, for example, the firing rate of Purkinje cells are correlated with both static (Frysinger et al., 1984) and dynamic force (Yamamoto et al., 2007). For movements of the eye, the instantaneous firing rate of floccular Purkinje cells can be quantitatively accounted for by the linear weighted sum of acceleration, velocity, and position of the eyes (Shidara et al., 1993).

While many movements require the contraction of an agonist set of muscles and the simultaneous relaxation of an antagonist set, there are tasks in which co-contraction of agonist and antagonist muscles are desirable such as when the co-contraction of the

opposing muscles is employed as a means to stiffen a limb. Thus while within the spinal cord there are known mechanisms in place that in part generate the reciprocal signals required for contraction and relaxation of corresponding muscles, the need for higher order task-dependent coordination is indispensable (De Luca and Mambrito, 1987). The cerebellar cortex is an optimal structure for coordinating the signals required for control of antagonist pair muscle activity. In support of this notion *in vivo* recordings from cerebellar Purkinje cells during motor tasks have demonstrated the presence of Purkinje cells whose activity are reciprocal to each other some of which change polarity as the direction of movement is reversed (Thach, 1970; Lisberger and Fuchs, 1978; Mano and Yamamoto, 1980; Frysinger et al., 1984). This latter finding is expected because movement in the reverse direction often requires the opposite action of the same group of muscles and thus the opposite control signal. These complementary Purkinje cell firing rates could very well be the mechanism by which cerebellum coordinates the activity of agonist and antagonist muscles pairs. The reciprocal firing rates generated by cerebellar cortical interneurons reported here might be one of the substrates with which the cerebellum generates these complementary signals. In this context, the stereotypic functional circuitry of the cerebellar cortex in the sagittal plane (Dizon and Khodakhah, 2011) provides for a simple substrate by which reciprocal activity of a complementary pair of Purkinje cells can be converted to one during which both Purkinje cells endorse the co-contraction of the muscle pairs. Dynamically, when co-contraction of agonist and antagonist muscle pairs is required activation of mossy fibers located immediately underneath both Purkinje cell pairs can ensure that the firing rate of both is increased concurrently.

It is worth emphasizing that the presence of the anatomical substrate for the generation of reciprocal signals does not necessarily mandate the presence of reciprocal signals under all conditions. For example reciprocally firing Purkinje cells are rarely found in the flocculus, uvula, and nodulus of mice and rabbit cerebella even though these regions have a similar cortical structure (De Zeeuw et al., 1995; Barmack and Yakhnitsa, 2008). The presence of neighboring Purkinje cells without reciprocal firing rates despite the presence of the anatomical substrate for generation of reciprocal circuitry suggests that the firing rate of one set of Purkinje cells is not always obligatorily coupled to decreases in the firing rate of another set of Purkinje cells. Dynamic or permanent changes in the spatial organization of active inputs, or plasticity could modify the functional output of the circuitry. For example, within the regions mentioned the granule cell layer accommodates numerous unipolar brush cells. These neurons directly receive mossy fiber inputs and excite laterally positioned granules cells (Takacs et al., 1999) thereby providing a mechanism for overriding the automatic generation of Purkinje cells reciprocal firing rates. Additionally, climbing-fiber mediated plasticity of both excitatory (Ito, 2001) and inhibitory (Mittmann and Hausser, 2007) inputs may silence the inputs to one set of Purkinje cells to effectively spare only one type of Purkinje cell response.

Clearly, the hypothesis that the reciprocal firing of groups of Purkinje cells control contraction of antagonist muscle pairs requires substantiation with experiments that simultaneously record EMG signals from against and antagonist muscles while simultaneously recording the activity of related Purkinje neurons. Moreover, while for the purposes of this discussion we have specifically considered cerebellar reciprocal signals within the context of coordination of agonist and antagonist muscles, our findings

on a mechanism for generation of these complementary signals are equally relevant to kinematic-based (Pasalar et al., 2006) models of cerebellar function. This is because in these models increases in the firing rate of one set of Purkinje cells coupled to the decrease of another set of Purkinje cells collectively encodes the population vector which predicts a limb's movement (Fortier et al., 1989).

As an additional mechanism to that described here, decreases in the firing rate of Purkinje cells may also be mediated through the action of climbing fibers on Purkinje cells (Davie et al., 2008; Mathy et al., 2009) or interneurons (Barmack and Yakhnitsa, 2008). While these mechanisms may act in concert with the granule cell → interneuron inhibitory pathway to decrease the firing rate of Purkinje cells, they are not necessary for the decreases to occur (Ke et al., 2009; Yakusheva et al., 2010).

The balance between excitation and inhibition in many brain regions may be the common underpinning that endows inhibition with the ability to perform multiple functions. For a single neuron, the balance but fixed delay between these two inputs dictates time windows for synaptic integration enabling the precise output of spikes (Pouille and Scanziani, 2001; Wehr and Zador, 2003; Mittmann et al., 2005). Moreover, inhibition prevents aberrant activity such as runaway excitation (Silberberg and Markram, 2007). Between neurons, excitation and inhibition shape the output of neurons located within specific regions of networks (Adesnik and Scanziani, 2010; Dizon and Khodakhah, 2011). Here, we have demonstrated that within these networks inhibition can scale appropriately with excitation. Thus, this canonical circuit may also mediate the generation of reciprocal activity for other brain regions such as motor control areas which

also operate as linear systems (Todorov, 2000; Ethier et al., 2006; Townsend et al., 2006; Bagnall et al., 2008).

REFERENCES

Adesnik H, Scanziani M (2010) Lateral competition for cortical space by layer-specific horizontal circuits. Nature 464:1155-1160.

Allen GI, Tsukahara N (1974) Cerebrocerebellar communication systems. Physiol Rev 54:957-1006.

Andersen P, Eccles JC, Voorhoeve PE (1964) Postsynaptic Inhibition of Cerebellar Purkinje Cells. J Neurophysiol 27:1138-1153.

Bagnall MW, McElvain LE, Faulstich M, du Lac S (2008) Frequency-independent synaptic transmission supports a linear vestibular behavior. Neuron 60:343-352.

Bao J, Reim K, Sakaba T (2010) Target-dependent feedforward inhibition mediated by short-term synaptic plasticity in the cerebellum. J Neurosci 30:8171-8179.

Barmack NH, Yakhnitsa V (2008) Functions of interneurons in mouse cerebellum. J Neurosci 28:1140-1152.

Calford MB, Semple MN (1995) Monaural inhibition in cat auditory cortex. J Neurophysiol 73:1876-1891.

Canepari M, Nelson L, Papageorgiou G, Corrie JE, Ogden D (2001) Photochemical and pharmacological evaluation of 7-nitroindolinyl-and 4-methoxy-7-nitroindolinyl-amino acids as novel, fast caged neurotransmitters. J Neurosci Methods 112:29-42.

Chadderton P, Margrie TW, Hausser M (2004) Integration of quanta in cerebellar granule cells during sensory processing. Nature 428:856-860.

Chan YS, Manzoni D, Pompeiano O (1982) Response characteristics of cerebellar dentate and lateral cortex neurons to sinusoidal stimulation of neck and labyrinth receptors. Neuroscience 7:2993-3011.

Davie JT, Clark BA, Hausser M (2008) The origin of the complex spike in cerebellar Purkinje cells. J Neurosci 28:7599-7609.

De Luca CJ, Mambrito B (1987) Voluntary control of motor units in human antagonist muscles: coactivation and reciprocal activation. J Neurophysiol 58:525-542.

De Zeeuw CI, Wylie DR, Stahl JS, Simpson JI (1995) Phase relations of Purkinje cells in the rabbit flocculus during compensatory eye movements. J Neurophysiol 74:2051-2064.

Dizon MJ, Khodakhah K (2011) The role of interneurons in shaping Purkinje cell responses in the cerebellar cortex. J Neurosci 31:10463-10473.

Eccles JC (1973) The cerebellum as a computer: patterns in space and time. J Physiol 229:1-32.

Eccles JC, Llinas R, Sasaki K (1966) The inhibitory interneurones within the cerebellar cortex. Exp Brain Res 1:1-16.

Ethier C, Brizzi L, Darling WG, Capaday C (2006) Linear summation of cat motor cortex outputs. J Neurosci 26:5574-5581.

Fortier PA, Kalaska JF, Smith AM (1989) Cerebellar neuronal activity related to whole-arm reaching movements in the monkey. J Neurophysiol 62:198-211.

Frysinger RC, Bourbonnais D, Kalaska JF, Smith AM (1984) Cerebellar cortical activity during antagonist cocontraction and reciprocal inhibition of forearm muscles. J Neurophysiol 51:32-49.

Fujita S (1967) Quantitative analysis of cell proliferation and differentiation in the cortex of the postnatal mouse cerebellum. J Cell Biol 32:277-287.

Gao W, Chen G, Reinert KC, Ebner TJ (2006) Cerebellar cortical molecular layer inhibition is organized in parasagittal zones. J Neurosci 26:8377-8387.

Georgopoulos AP, Schwartz AB, Kettner RE (1986) Neuronal population coding of movement direction. Science 233:1416-1419.

Hausser M, Clark BA (1997) Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. Neuron 19:665-678.

Ito M (2001) Cerebellar long-term depression: characterization, signal transduction, and functional roles. Physiol Rev 81:1143-1195.

Jaeger D, Bower JM (1994) Prolonged responses in rat cerebellar Purkinje cells following activation of the granule cell layer: an intracellular in vitro and in vivo investigation. Exp Brain Res 100:200-214.

Ke MC, Guo CC, Raymond JL (2009) Elimination of climbing fiber instructive signals during motor learning. Nat Neurosci 12:1171-1179.

Lisberger SG, Fuchs AF (1978) Role of primate flocculus during rapid behavioral modification of vestibuloocular reflex. I. Purkinje cell activity during visually guided horizontal smooth-pursuit eye movements and passive head rotation. J Neurophysiol 41:733-763.

Lisberger SG, Pavelko TA (1986) Vestibular signals carried by pathways subserving plasticity of the vestibulo-ocular reflex in monkeys. J Neurosci 6:346-354.

Llinas R, Sugimori M (1980) Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. J Physiol 305:171-195.

Manni E, Dow RS (1963) Some Observations on the Effects of Cerebellectomy in the Rat. J Comp Neurol 121:189-194.

Mano N, Yamamoto K (1980) Simple-spike activity of cerebellar Purkinje cells related to visually guided wrist tracking movement in the monkey. J Neurophysiol 43:713-728.

Marshall SP, Lang EJ (2009) Local changes in the excitability of the cerebellar cortex produce spatially restricted changes in complex spike synchrony. J Neurosci 29:14352-14362.

Mastronarde DN (1983) Correlated firing of cat retinal ganglion cells. II. Responses of X- and Y-cells to single quantal events. J Neurophysiol 49:325-349.

Mathy A, Ho SS, Davie JT, Duguid IC, Clark BA, Hausser M (2009) Encoding of oscillations by axonal bursts in inferior olive neurons. Neuron 62:388-399.

McKay BE, Turner RW (2005) Physiological and morphological development of the rat cerebellar Purkinje cell. J Physiol 567:829-850.

Medina JF, Lisberger SG (2007) Variation, signal, and noise in cerebellar sensory-motor processing for smooth-pursuit eye movements. J Neurosci 27:6832-6842.

Medina JF, Lisberger SG (2008) Links from complex spikes to local plasticity and motor learning in the cerebellum of awake-behaving monkeys. Nat Neurosci 11:1185-1192.

Mittmann W, Hausser M (2007) Linking synaptic plasticity and spike output at excitatory and inhibitory synapses onto cerebellar Purkinje cells. J Neurosci 27:5559-5570.

Mittmann W, Koch U, Hausser M (2005) Feed-forward inhibition shapes the spike output of cerebellar Purkinje cells. J Physiol 563:369-378.

Miyashita Y, Nagao S (1984) Contribution of cerebellar intracortical inhibition to Purkinje cell response during vestibulo-ocular reflex of alert rabbits. J Physiol 351:251-262.

Moore CI, Nelson SB (1998) Spatio-temporal subthreshold receptive fields in the vibrissa representation of rat primary somatosensory cortex. J Neurophysiol 80:2882-2892.

Pasalar S, Roitman AV, Durfee WK, Ebner TJ (2006) Force field effects on cerebellar Purkinje cell discharge with implications for internal models. Nat Neurosci 9:1404-1411.

Pouille F, Scanziani M (2001) Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. Science 293:1159-1163.

Pouzat C, Hestrin S (1997) Developmental regulation of basket/stellate cell-->Purkinje cell synapses in the cerebellum. J Neurosci 17:9104-9112.

Priebe NJ, Ferster D (2008) Inhibition, spike threshold, and stimulus selectivity in primary visual cortex. Neuron 57:482-497.

Raman IM, Bean BP (1999) Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. J Neurosci 19:1663-1674.

Robinson FR, Fuchs AF (2001) The role of the cerebellum in voluntary eye movements. Annu Rev Neurosci 24:981-1004.

Sakaba T (2008) Two Ca(2+)-dependent steps controlling synaptic vesicle fusion and replenishment at the cerebellar basket cell terminal. Neuron 57:406-419.

Shidara M, Kawano K, Gomi H, Kawato M (1993) Inverse-dynamics model eye movement control by Purkinje cells in the cerebellum. Nature 365:50-52.

Shin SL, Hoebeek FE, Schonewille M, De Zeeuw CI, Aertsen A, De Schutter E (2007) Regular patterns in cerebellar Purkinje cell simple spike trains. PLoS One 2:e485.

Silberberg G, Markram H (2007) Disynaptic inhibition between neocortical pyramidal cells mediated by Martinotti cells. Neuron 53:735-746.

Steuber V, Mittmann W, Hoebeek FE, Silver RA, De Zeeuw CI, Hausser M, De Schutter E (2007) Cerebellar LTD and pattern recognition by Purkinje cells. Neuron 54:121-136.

Takacs J, Markova L, Borostyankoi Z, Gorcs TJ, Hamori J (1999) Metabotrop glutamate receptor type 1a expressing unipolar brush cells in the cerebellar cortex of different species: a comparative quantitative study. J Neurosci Res 55:733-748.

Thach WT (1970) Discharge of cerebellar neurons related to two maintained postures and two prompt movements. II. Purkinje cell output and input. J Neurophysiol 33:537-547.

Todorov E (2000) Direct cortical control of muscle activation in voluntary arm movements: a model. Nat Neurosci 3:391-398.

Townsend BR, Paninski L, Lemon RN (2006) Linear encoding of muscle activity in primary motor cortex and cerebellum. J Neurophysiol 96:2578-2592.

Vincent P, Armstrong CM, Marty A (1992) Inhibitory synaptic currents in rat cerebellar Purkinje cells: modulation by postsynaptic depolarization. J Physiol 456:453-471.

Walter JT, Khodakhah K (2006) The linear computational algorithm of cerebellar Purkinje cells. J Neurosci 26:12861-12872.

Walter JT, Khodakhah K (2009) The advantages of linear information processing for cerebellar computation. Proc Natl Acad Sci U S A 106:4471-4476.

Walter JT, Dizon MJ, Khodakhah K (2009) The functional equivalence of ascending and parallel fiber inputs in cerebellar computation. J Neurosci 29:8462-8473.

Wehr M, Zador AM (2003) Balanced inhibition underlies tuning and sharpens spike timing in auditory cortex. Nature 426:442-446.

Wilson RI, Mainen ZF (2006) Early events in olfactory processing. Annu Rev Neurosci 29:163-201.

Wisden W, Murray AJ, McClure C, Wulff P (2009) Studying Cerebellar Circuits by Remote Control of Selected Neuronal Types with GABA(A) Receptors. Front Mol Neurosci 2:29.

Yakusheva T, Blazquez PM, Angelaki DE (2010) Relationship between complex and simple spike activity in macaque caudal vermis during three-dimensional vestibular stimulation. J Neurosci 30:8111-8126.

Yamamoto K, Kawato M, Kotosaka S, Kitazawa S (2007) Encoding of movement dynamics by Purkinje cell simple spike activity during fast arm movements under resistive and assistive force fields. J Neurophysiol 97:1588-1599.

FIGURES

FIGURE 1

Activity of single patches of granule cells can drive the reciprocal firing rates of laterally positioned Purkinje cells

(A) Top: Direct input from a single patch of granule cells (depicted as red cells) provides net excitation (red zone) to Purkinje cells (depicted in black). Feed-forward inhibition by interneurons (green and yellow cells) also allows the same patch of granule cells to inhibit a second group of Purkinje cells (blue zone). (i) A pair of Purkinje cells were monitored extracellularly in response to photo-release of glutamate on a single patch of granule cells. (ii) Raster plot of the activity of the same two cells in response to varying stimulus strengths.

(B) The maximum and minimum instantaneous firing rates (each trial: open circles; mean±S.D.: closed circles) of the Purkinje cell pair recorded in A as a function of stimulation strength (photolysis power). Solid line represents the linear regression (red: $R = 0.68$, $P = 0.002$; blue: $R = -0.41$, $P = 0.088$). Dotted line: baseline firing rate of each cell = 46.4 sp/s and 36.4 sp/s.

(C) The average (±S.E.M) minimum instantaneous firing rate of all 5 Purkinje cells examined as a function of photolysis power. Solid line represents the linear regression (red: $R = 0.99$, $P < 0.001$; blue: $R = -0.94$, $P = 0.018$). The position of the symbols on the ordinate indicate the average baseline firing rate of cells receiving excitation (41.9 ± 2.1 sp/s) and inhibition (38.5 ± 0.7 sp/s) (unpaired t-test: $P = 0.24$).

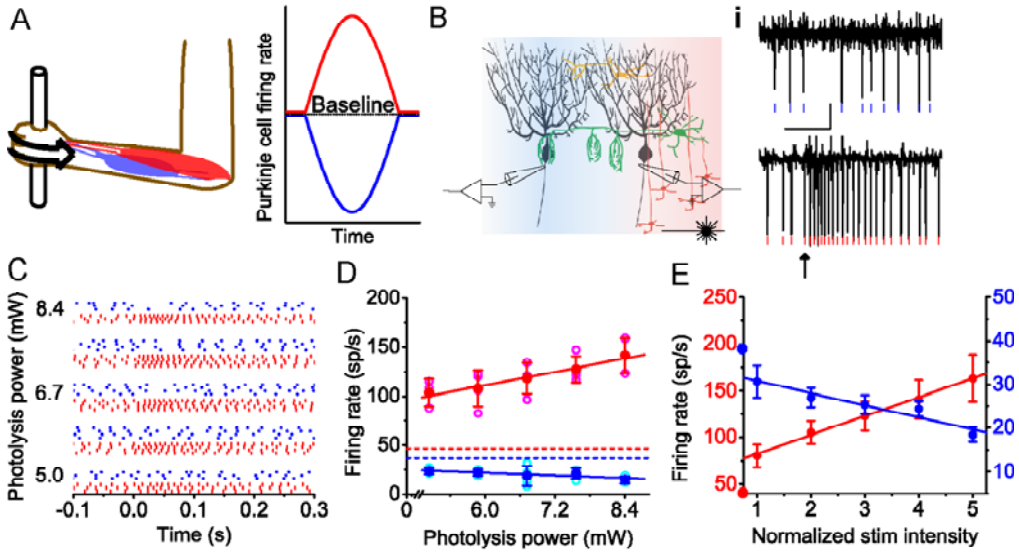

A
Baseline
Purkinje cell firing rate
Time
B
i
C
Photolysis power (mW)
8.4
6.7
5.0
-0.1
0.0
0.1
0.2
0.3
Time (s)
D
Firing rate (sp/s)
200
150
100
50
0
6.0
7.2
8.4
Photolysis power (mW)
E
Firing rate (sp/s)
250
200
150
100
50
50
40
30
20
10
1
2
3
4
5
Normalized stim intensity

FIGURE 2

Feed-forward inhibition enables Purkinje cells to linearly encode the strength of granule cell inputs in their firing rate

(A) Left: Response of an extracellularly monitored Purkinje cell to photo-activation of a patch of granule cells by glutamate photolysis at two different laser powers. The location of the activated patch of granule cells was chosen to inhibit the activity of the target Purkinje cell. Scale bar: 50 ms, 100 μV. Right, raster plot of responses of the same cell to varying stimulations strengths (@ t=0).

(B) The minimum instantaneous firing rate (each trial: gray circles; mean±S.D.: black circles) of the Purkinje cell recorded in A as a function of photolysis power. Solid line represents the linear regression. Baseline firing rate = 37.0 sp/s.

(C) The change in the post-response number of spikes (each trial: gray circles; mean±S.D.: black circles) of the Purkinje cell recorded in A as a function of photolysis power. Solid line represents the linear regression.

(D) The reduction in the number of spikes fired by the cell following activation of a patch of granule cells is a linear function of its minimum instantaneous firing rate**,** mean±S.D.: black squares. Solid line: linear regression ($R = 0.98$, $P < 0.0001$).

(E) The average (±S.E.M) minimum instantaneous firing rate of all 14 Purkinje cells examined as a function of photolysis power. Solid line represents the linear regression. Average baseline firing rate = 40.4 ± 2.1 sp/s.
(F) The average change (n=14 cells) in the number of spikes fired post-stimulus by Purkinje cells is a linear function of the average minimum instantaneous firing rate in the same cells, mean±S.E.M.: black squares. Solid line: linear regression ($R = 0.98$ $P < 0.0001$).

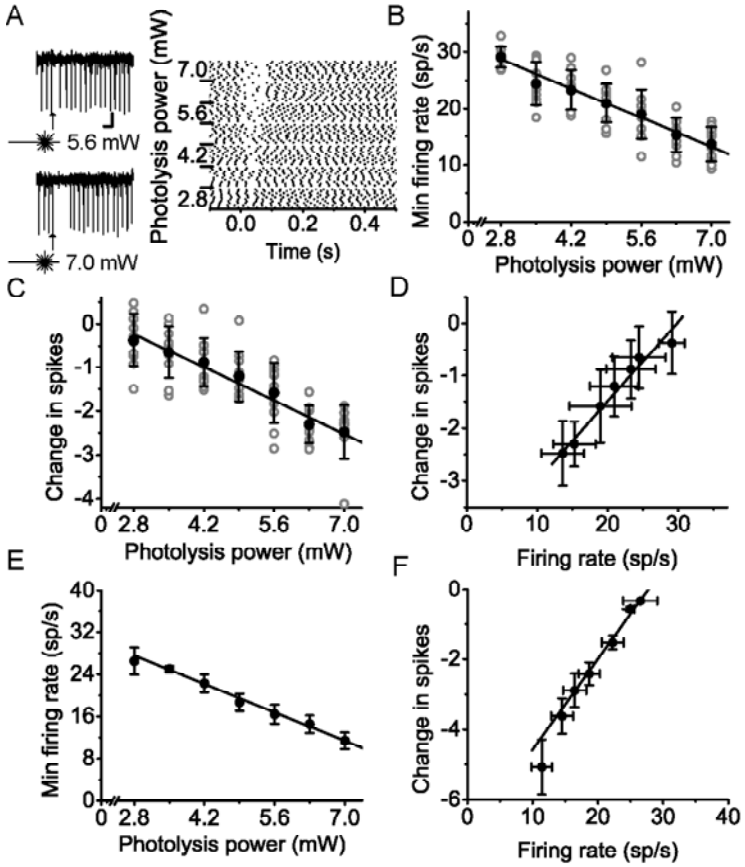

A
5.6 mW
7.0 mW
Photolysis power (mW)
7.0
5.6
4.2
2.8
0.0
0.2
0.4
Time (s)
B
Min firing rate (sp/s)
Photolysis power (mW)
C
Change in spikes
Photolysis power (mW)
D
Change in spikes
Firing rate (sp/s)
E
Min firing rate (sp/s)
Photolysis power (mW)
F
Change in spikes
Firing rate (sp/s)

FIGURE 3

Purkinje cell firing rate linearly encodes the strength of granule cell inputs *via* inhibition *in vivo*.

(A) Response of an extracellularly recorded Purkinje cell *in vivo* to electrical stimulation of a lateral patch of granule cells (3 stimuli @100Hz, each consisting of a 200 μs, 40 μA pulse stimulus is denoted by the gray rectangle) in the (i) absence and (ii) presence of a $GABA_A$ blocker. Scale bar: 100 ms, 200 μV. Bottom: The bottom panel shows raster plots of the same cell at various stimulus strengths.

(B) The minimum instantaneous firing rate (each trial: gray circles; mean±S.D.: black circles) of the Purkinje cell recorded in A as a function of the stimulus intensity. Solid line represents the linear regression ($R = -0.80$, $P < 0.0001$). Baseline firing rate = 35.2 spikes per second.

(C) The average (±S.E.M) minimum instantaneous firing rate of all 5 Purkinje cells examined as a function of photolysis power. Solid line represents the linear regression ($R = -0.98$, $P = 0.003$). Average baseline firing rate = 50.6 ± 4.6 sp/s.

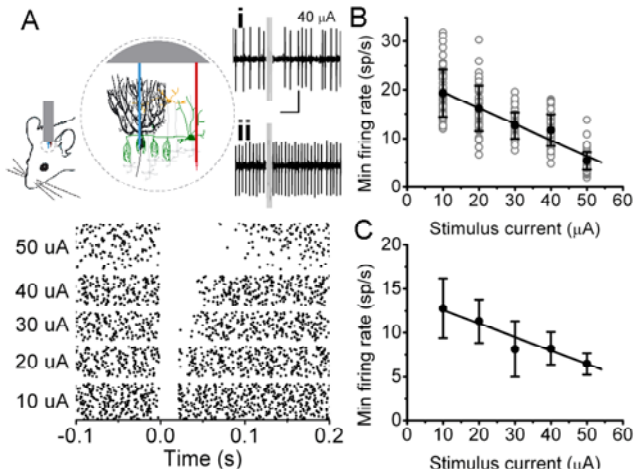

A
i
40 μA
ii
50 uA
40 uA
30 uA
20 uA
10 uA
-0.1
0.0
0.1
0.2
Time (s)
B
Min firing rate (sp/s)
0
10
20
30
40
50
60
Stimulus current (μA)
C
Min firing rate (sp/s)
0
5
10
15
20
Stimulus current (μA)

FIGURE 4

Feed-forward inhibition is mediated by linear input-output components.

(A,B) A patch of granule cells positioned immediately underneath an interneuron was photo-activated while the activity of the target interneuron was monitored extracellularly. The concentration of photoreleased glutamate was varied by altering the laser power to change the strength of granule cell inputs. Raw data scale bar: 100 ms, 50 μV. In the raster plots the stimulus was delivered at t=0.

(C) The maximum firing rate of the interneuron recorded in B (gray circles; mean±S.D.: black circles) and the average data from 10 interneurons (mean±S.E.M.: black squares) Solid lines: linear regression. Dotted line: the average baseline firing rate (9.69 ± 2.13 sp/s).

(D,E) Synaptically-evoked GABAergic changes produced by photolytic activation of a patch of granule cells were measured in a voltage-clamped Purkinje cell ($V_{Command}$ = 0 mV). Black traces show the single trial GABAergic conductances, whereas the top set of colored traces show averaged conductances at photolysis powers indicated in B. Scale bar: 100 ms and 2 nS. The lower set of colored traces is normalized to allow comparison of their kinetics (scale bar: 50 ms).

(F) Average (±S.E.M.) of GABAergic conductance areas plotted as a function of photolysis power. Solid line: linear regression.

(G) The response of the same Purkinje cell to varying strengths of activation of a patch of granule cells was first recorded extracellularly, and then under voltage-clamp ($V_{Command}$ = 0 mV).

(H) Post-stimulus time histogram of a cell examined as detailed in G to varying strength of granule cell input with its corresponding GABA conductance traces overlaid. Stimulus was delivered at t=0. Y-scale: 65 sp/s and 18 nS.

(I) Average Purkinje cell minimum firing rate as a function of the average GABA conductance area (n=5 cells) obtained from experiments outlined in G,H.

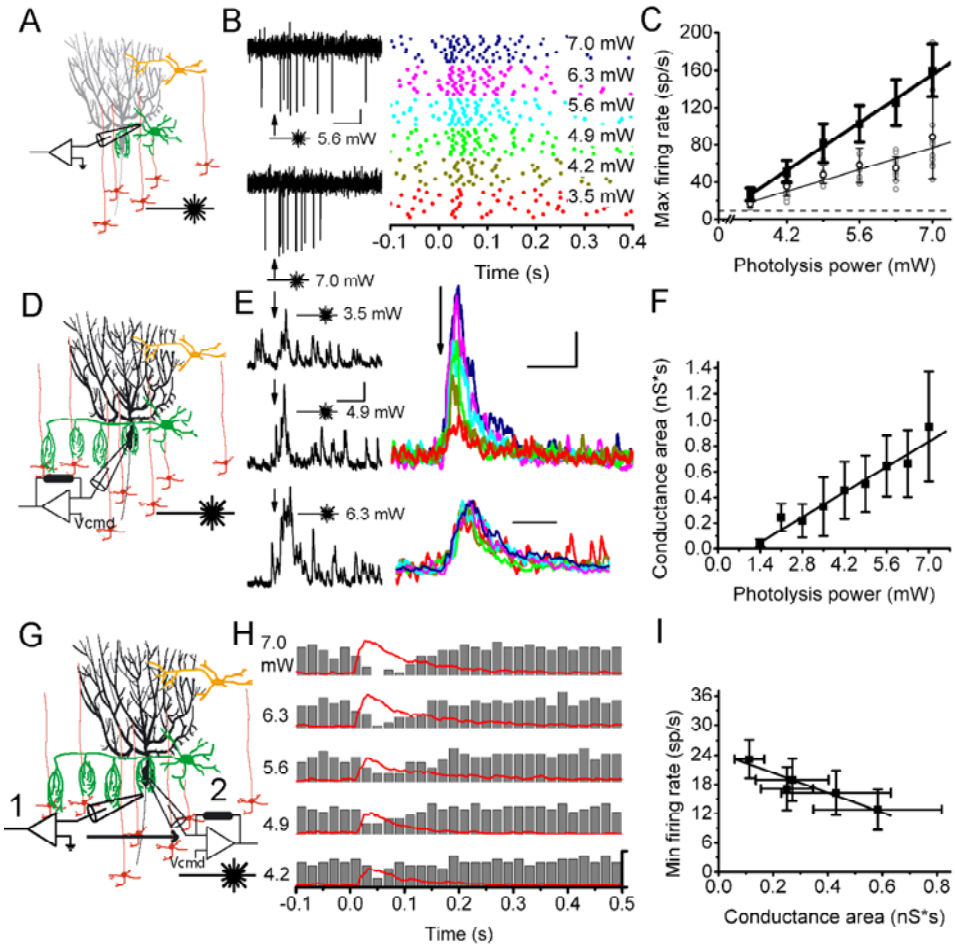
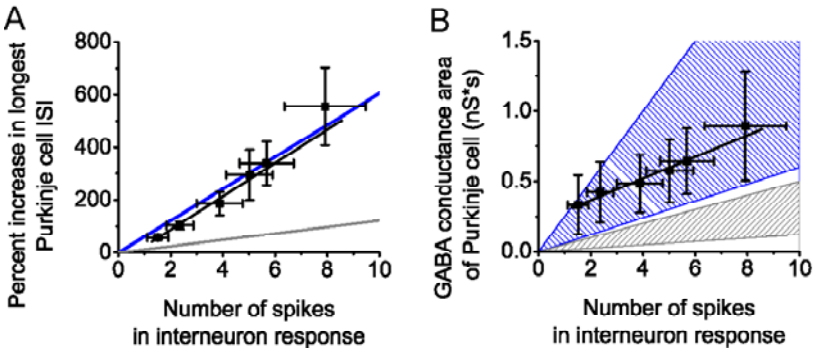

A
B
C
5.6 mW
7.0 mW
6.3 mW
5.6 mW
4.9 mW
4.2 mW
3.5 mW
-0.1 0.0 0.1 0.2 0.3 0.4
Time (s)
Max firing rate (sp/s)
200
160
120
80
40
0
0 4.2 5.6 7.0
Photolysis power (mW)
D
E
F
7.0 mW
3.5 mW
4.9 mW
6.3 mW
Vcmd
Conductance area (nS*s)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0 1.4 2.8 4.2 5.6 7.0
Photolysis power (mW)
G
H
I
1
2
Vcmd
7.0 mW
6.3
5.6
4.9
4.2
-0.1 0.0 0.1 0.2 0.3 0.4 0.5
Time (s)
Min firing rate (sp/s)
36
30
24
18
12
6
0
0.0 0.2 0.4 0.6 0.8
Conductance area (nS*s)

FIGURE 5

The GABA conductance evoked in Purkinje cells by activation of a patch of granule cells is composed of the concerted activity of ~5 interneurons

(A) The estimated number of interneurons activated by photorelease of glutamate, which contributed to inhibition of Purkinje cells under our experimental conditions. The number of interneurons was estimated by extrapolating data from Hausser and Clark (1997), which demonstrated that a single interneuron prolongs the interspike interval of Purkinje cells by ~12% (see text for specifics on the method used for extrapolation).

(B) The estimated number of interneurons activated under our experimental conditions using data from two published works. Vincent et al. (1992) and Pouzat and Hestrin (1997) characterized the interneuron-Purkinje cell IPSC. The number of interneurons was approximated by the parameters of the IPSCs found in these two investigations (see text for specifics on the method of extrapolation).


A
Percent increase in longest Purkinje cell ISI
800
600
400
200
0
0
2
4
6
8
10
Number of spikes in interneuron response
B
GABA conductance area of Purkinje cell (nS*s)
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Number of spikes in interneuron response