İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IN VITRO FUNCTIONAL ANALYSIS OF SRY RELATED TRANSCRIPTION FACTOR SOX11 IN ADULT NEUROGENESIS

M.Sc. Thesis by
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Department : **Advanced Technologies**

Programme: **Molecular Biology and Biotechnology**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SRY İLİŞKİLİ TRANSKRİPSYON FAKTÖRÜ SOX11'İN ERİŞKİN NÖROJENEZDEKİ ROLÜNÜN HÜCRE KÜLTÜRÜNDE ARAŞTIRILMASI

YÜKSEK LİSANS TEZİ

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TABLE OF CONTENTS

ABBREVIATIONS

AHPs : Adult hippocampal stem/ progenitor cells
Amp : Ampicillin
APS : Amonium persulfate
ATP : Adenosine triphosphate
BrdU : 5-bromo-2-deoxyuridine
BSA : Bovine serum albumin
bp : Base pair
CA3 : Ammon's horn or *cornu ammonis* of the hippocampus
cDNA : Copy DNA
CNS : Central nervous system
Cy 3 : Carbocyanin
Cy 5 : indodicarbocyanin
DCX : Doublecortin
DG : Dentate gyrus
DMEM : Dulbecco's modified eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxy nucletodide triphosphat
dsDNA : Double strand DNA
DTT : Dithiothreitol
E. coli : Escherichia coli
ECL : Enhanced chemiluminescent
EDTA : Ethylenediamine tetraacetic acid
EGTA : Ethylene glycol tetraacetic acid
FGF2 : Fibroblast growth factor 2
FITC : Fluorescein isothiocyanate
Fsk : Forskolin
GCL : Granule cell layer
GFAP : Glial fibrillary acidic protein
GFP : Green fluorescent protein
h : Hour/hours
HEK : Human embryonic kidney
HMG : High mobility group
HRP : Horseradish peroxidase
kb : Kilo base pair
kDa : kilo Dalton
LB : Luria Bertain
M : Molar
MCS : Multiple cloning site
min : Minute
mRNA : messenger RNA

MW : Molecular weight
NSC : Neural stem cell
OB : Olfactory bulb
P-Orn : Poly-Ornithine
PB : Phosphate buffer
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
PFA : Paraformaldehyde
PSA-NCAM : Polysialylated form of the neural cell adhesion molecule
PGL : Periglomerular layer
POU : Pit-Oct-Unc
PSF : Penicillin, streptomycin, fungizone
PVDF : Polyvinylidenfluorid
rcf : Relative centrifugation force
RMS : Rostral migratory stream
RNA : Ribonucleic acid
rpm : Rounds per minute
RT PCR : Reverse Transcriptase PCR
SDS : Sodium dodecyl sulfate
sec : Second
SGZ : Subgranular zone
shRNA : Short hairpin RNA
Sry : Sex-determining Region Y
Sox : Sry related HMG box transcription factor
SVZ : Subventricular zone
TAE : Tris acetate buffer/EDTA
TBS : Tris buffered saline
TBST : Tris buffered Saline/Tween
TEMED : Tetramethylethylendiamin
TNE : Tris-HCl/NaCl/EDTA
Tris : Trishydroxymethylaminomethan
Tuj1 : Class III beta tubulin
UV : Ultraviolet

LIST OF TABLES

LIST OF FIGURES

In vitro Functional Analysis of Sry Related Transcription Factor Sox11 in Adult Neurogenesis

SUMMARY

The generation of new neurons from neural stem cells is restricted to two regions in the adult mammalian brain: the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. In both regions, adult neurogenesis encompasses different steps including activation of quiescent stem cells, proliferation of stem and progenitor cells, neuronal fate commitment and maturation and integration of newly developed neurons. Extrinsic and intrinsic pathways regulate all these stages of adult neurogenesis. Sets of transcription factors from different families act together to control cell fate decisions of progenitor cells and differentiation of precursors into each lineage.

In this study Sry related high-mobility-group box (Sox) transcription factor 11 is focused on to understand its role in adult neurogenesis. Initially, Sox11 expression in adult neurogenic lineages was investigated. Sox11 expression was detected in neurogenic lineages of adult mice brain in combination of identified cellular markers. It was shown that Sox11 expressing cells do not express Sox2 or GFAP that are neural stem cell markers and Sox11 expressing cells also express immature neuronal markers like doublecortin (DCX) and beta III tubulin (Tuj1). As a conclusion it was shown in this study that Sox11 is expressed in immature neuronal precursors of adult rostral migratory stream and dentate gyrus. To answer the question whether Sox11 play a role in immature neuronal precursors of adult neurogenic lineages, Sox11 *in vitro* gain of function analysis was performed. Sox11 was overexpressed in the cultured adult hippocampal stem/progenitor cells (AHPs) derived from adult rats by electroporation and viral transduction of overexpression cassette. These adult rat hippocampal progenitors were kept under two separate differentiation conditions for four days. Both AHPs were then analyzed with the expression of immature neuronal marker doublecortin. It was shown that doublecortin (DCX) expression was induced in Sox11 overexpressing cells. This observation indicates that Sox11 overexpression promotes neuronal differentiation of adult hippocampal progenitors *in vitro*. Additionally in this study Sox11 effect on DCX promoter was investigated in human embryonic kidney (HEK) cells via luciferase assay. Even very little amount of Sox11 (8ng/30x10^6 cells) highly induced (50 folds) DCX expression. This result gives the idea that Sox11 is an upstream element of DCX promoter. Finally, short hairpin RNAs (shRNAs) against Sox11 was produced to generate a tool for loss of function analysis to clarify its role in adult neurogenesis. Four different target sequences were selected inside the mouse Sox11 coding sequence. Four different shRNA were synthesized depending on the selected sequences. Afterwards functionality of

Sox11-shRNAs was tested for further approaches via Western blot and psi-CHECK^{TM} luciferase assay system. It was shown that two of the produced shRNAs were functional and they could be used for loss of function analyses.Sry İlişkili Transkripsyon Faktörü Sox11'in Erişkin Nörojenezdeki Rolünün Hücre Kültüründe Araştırılması

Sry İlişkili Transkripsyon Faktörü Sox11'in Erişkin Nörojenezdeki Rolünün Hücre Kültüründe Araştırılması

ÖZET

Yetişkin memeli beyninde yeni sinir hücreleri sadece subventriküler alanın ventrikül yatay duvarında ve hipokampusün subgranüler kısmında oluşturulmaktadır. Bu iki kısımda da gerçekleşen nörojenez; sessiz kök hücrelerinin aktifleştirilmesi, kök hücre ve projenitör hücrelerin proliferasyonu, nöronal kaderin seçilmesi ve yeni gelişmiş sinir hücrelerinin entegrasyonu adımlarından oluşmaktadır. Yetişkinlerdeki nörojenezin tüm bu adımları içsel ve dışsal yol izleriyle kontrol edilmektedir. Farklı ailelerden transkripsyon faktörleri projenitör hücrelerin kader seçiminde ve farklı hücre soylarına diferansiye olmalarında birlikte etkili olmaktadır.

Bu çalışmada Sry ilişkili transkripsyon faktörü Sox11'in yetişkin nörojenezdeki rolünün anlaşılması amaçlanmıştır. Öncelikle Sox11 proteininin yetişkin nörojenik alanlardaki ekspresyonu araştırıldı. Bunun için farklı hücresel markırlar kullanılarak yetişkin fare beyninde nörojenik alanlardaki Sox11 protein ekspresyonu tespit edildi. Bu çalışmada Sox11 sentezleyen hücrelerin nöral kök hücre markırları olan Sox2 ve GFAP'yi sentezlemediği fakat olgunlaşmamış nöron markırlarından doublekortin (DCX) ve beta III tübülini (Tuj1) sentezlediği belirlenmiştir. Sox11'in rostral göç yolunda ve hipokampuste sadece olgunlaşmamış nöronal precursor hücrelerde ekspresyonu gözlendi. Buradan yola çıkarak Sox11'in yetişkin nörojenik alanlardaki olgunlaşmamış nöronal sinir hücrelerinde nasıl bir fonksyonu olduğunu anlamak için Sox11 gen ifadesi hücre kültüründeki yetişkin hipokampus hücrelerinde iki ayrı sistem ile fazlaca arttırıldı. Sox11 ekspresyon kaseti yetişkin sıçan hipokampüs hücre kültürüne elektroporasyon ve viral transdüksyon yolu ile gönderildi. Bu yetişkin sıçan hipokampüs projenitör hücreleri dört gün süresince iki farklı nöronal diferansiyasyon kondisonunda tutuldu. Her iki diferansiyasyon şartlarındaki Sox11'i fazlaca eksprese eden yetişkin hücrelerde, olgunlaşmamış nöronal markır doublekortinin ekspresyonu incelendi ve bu hücrelerde doublekortin ekspresyonunun arttığı gözlendi. Bu çalışma, Sox11 gen ifadesinin arttırılmasıyla yetişkin hipokampüs hücrelerinin nöronal farklılaşmasının arttığını göstermiştir. Bu çalışmada ayrıca Sox11'in DCX promotor bölgesine olan etkisi insan embryonik böbrek hücre kültüründe lusiferaz ölçümü ile incelendi. Çok az miktardaki Sox11 (8ng/30x10^6 hücre)'in bile DCX promotor aktivitesini yüksek oranda (50 kat) arttırdığı gözlendi. Son olarak, Sox11'in yetişkinlerde nörojenik alanlardaki rolünü aydınlatmak için Sox11'in gen ifadesini susturacak Sox11'e spesifik RNA interferanz molekülleri sentezletildi. Sox11'in anlamlı dizisi içerisinden dört farklı hedef sekansı seçildi ve onlara bağlı olarak RNA

interferanz molekülleri sentezletildi. Western blot ve psi-CHECK^{TM} lusiferaz sistemleriyle hazırlanmış RNA interferanz dizilerinin Sox11'e ne kadar spesifik ve fonksyonel oldukları araştırıldı. Bunlardan iki tanesinin hücrede fonksyonel olarak Sox11'i susturduğu gösterildi.

1. INTRODUCTION

1.1 Adult neurogenesis

Stem cells that have the potential to develop into different specialized tissue cells are one of the most promising areas of science in terms of their clinical potential. Embryonic stem cells from the inner cell mass of the blastula are multipotent cells that give rise to all body tissues except placenta. Stem cells in some of the adult tissues like blood, bone and epithelia, have the ability to renew themselves and to replace the tissue cells with the new ones under certain physiological conditions (Morrison and Spradling, 2008). Since stem cells have high potential repair capacity, cell based therapies using these stem cells to treat diseases are in great importance.

Although in the traditional view of the brain after birth is considered post-mitotic, it was a great wonder if there are stem cells in adult central nervous system. Neural stem cells (NCS) and restricted progenitors are both found in many regions of the central nervous system (CNS) throughout life whereas it was a central dogma that 'no new neuron is added to adult brain' till 1965. Embryonic neural stem cells (NSCs) that give rise to neuroepithelial cells arise from ectoderm. The neuroepithelial cells then produce radial glia that generates fetal and adult NSCs in the CNS. Stem cells in the CNS are 'self-renewing' to produce indistinguishable progeny from themselves, 'proliferative' to undergo continuously mitotic division and 'multipotent' to generate neurons and glia lineages i.e. astrocytes, oligodendrocytes (Weiner, 2007). Neurogenesis, producing functionally integrated neurons from progenitors was believed to occur only in the embryo (Ramon y Cajal, 1913) In 1967, first evidence that neurogenesis occurs in mature rat and guinea pig brain was shown (Altman and Das). Neurogenesis in adult monkeys (Gould *et al.*, 1999) and then in the human hippocampus (Eriksson *et al.*, 1998) was demonstrated with the generated technique of labeling the dividing neurons during S phase with a synthetic thymidine analogue bromodeoxyuridine (BrdU) and detecting the labeled

cells by immunohistochemistry. Nowadays it is generally accepted that neurogenesis occurs in distinct parts of the adult brain (Gross CG, 2000).

Neurogenesis actively occurs throughout the life of most mammals in subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Lie *et al.*, 2004) (Fig 1.1).

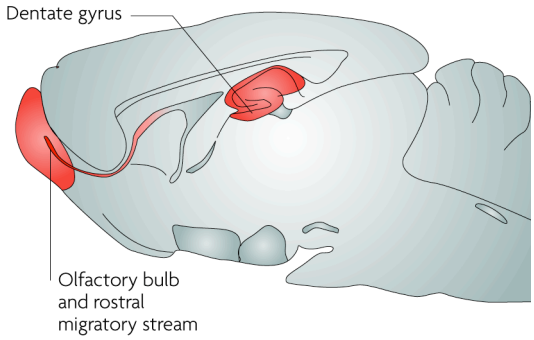


Figure 1.1: Discrete regions that neurogenesis is active in the adult mammalian brain (Elizabeth Gould, 2007).

NSCs in the SVZ of the lateral ventricles generate transiently amplifying cells. They differentiate into immature neurons that migrate over a great distance with each other in chains through the rostral migratory stream (RMS) to the olfactory bulb. The migrating neurons are covered by astrocytes. After the immature neurons reach to the olfactory bulb, new neurons migrate radially to the outer layers of the olfactory bulb. Immature neurons differentiate into granule neurons and periglomerular neurons (Ming and Song, 2005).

NSCs in the SGZ of the hippocampus that have radial processes projected through the granular cell layer (GCL) and tangential processes extended to the GCL and the hilus give rise to transiently amplifying progenitors. Those cells then differentiate into immature neurons that migrate into the GCL where they become dentate granule cells. Migrated immature neurons extend their axonal projections along the mossy fiber pathway to the Ammon's horn or *cornu ammonis* (CA3) of the hippocampus pyramidal cell layer and their dendrites toward the molecular layer. Newly developed granule neurons then integrate into the neuronal network (Ming and Song, 2005).

1.2 Markers of neurogenesis

Developmental stages of adult neurogenesis are identified with expression of unique sets of markers and morphology differences between cells. This fundamental information is very important for learning origins of new neurons and further clinical applications.

Olfactory bulb (OB) neurogenesis initiators are located in the lateral walls of the lateral ventricles in the SVZ. These cells are slowly proliferating neuronal precursor type B cells with astrocytic properties like glial fibrillary acidic protein (GFAP) positive intermediate filaments, light cytoplasm, gap junctions, and glycogen granules. These cells give rise to transiently amplifying type C cells in the SVZ (Doetsch *et al.*, 1997). Type C cells then form type A migrating neuroblasts. Type A cells migrate from the lateral walls through the RMS to the OB in chains that are covered by type B cells (Alvarez-Buylla *et al.*, 2002). Type A cells can be identified by doublecortin (DCX) and polysialylated form of the neural cell adhesion molecule (PSA-NCAM) expression that are both associated with neuronal migration. Neuron specific class III beta tubulin (Tuj1) is also expressed in those immature neurons (Kempermann, 2006). During the migration along the RMS, type A cells continue to divide and initiate neuronal maturation and a few of type A cells become calretinin positive (Jankovski and Sotelo, 1996). When they reach to the OB, they differentiate into mature neurons that express NeuN and calretinin.

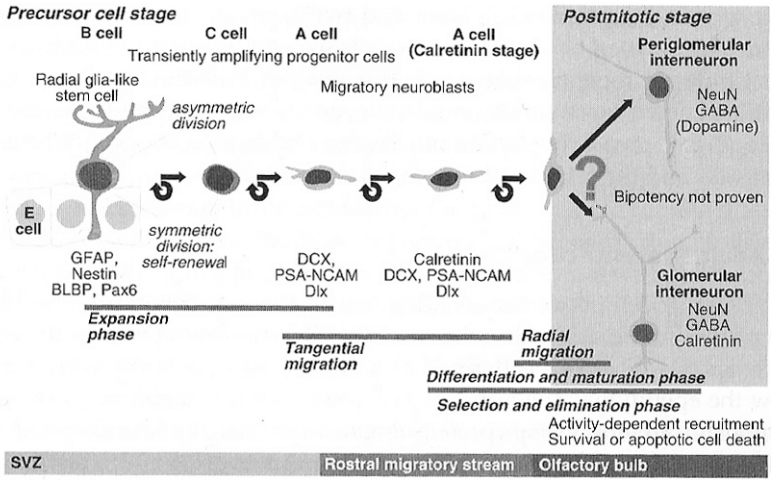


Figure 1.2: Model of adult neurogenesis in SVZ (Kempermann, Adult Neurogenesis pg: 148).

In the adult SGZ similar to SVZ, primary progenitor type 1 cells have triangular-shaped soma, radial glia like morphology with long apical processes and astrocytic properties like GFAP expression (Seri *et al.*, 2001). Type 1 cells give rise to transiently amplifying progenitor type 2 cells. Type 2 cells express nestin similar to type 1 cells but do not express GFAP. They have short processes located more or less parallel to the SGZ. They are classified under two groups such as DCX negative type 2a and DCX positive type 2b (Kronenberg *et al.*, 2003). Type 2b cells have both properties of precursor cells expressing nestin and lineage determined immature neurons expressing DCX and PSA-NCAM. Afterwards type 2 cells generate type 3 migrating neuroblasts that are located horizontally or more vertically to the SGZ. Type 3 cells that express DCX but do not express nestin radially migrate into the granular cell layer. Type 3 post-mitotic cells that are characterized by calretinin expression start to differentiate into granule cells (Kempermann *et al.*, 2003). When they become mature granule cells, calretinin is converted to calbindin expression (Brandt *et al.*, 2003).

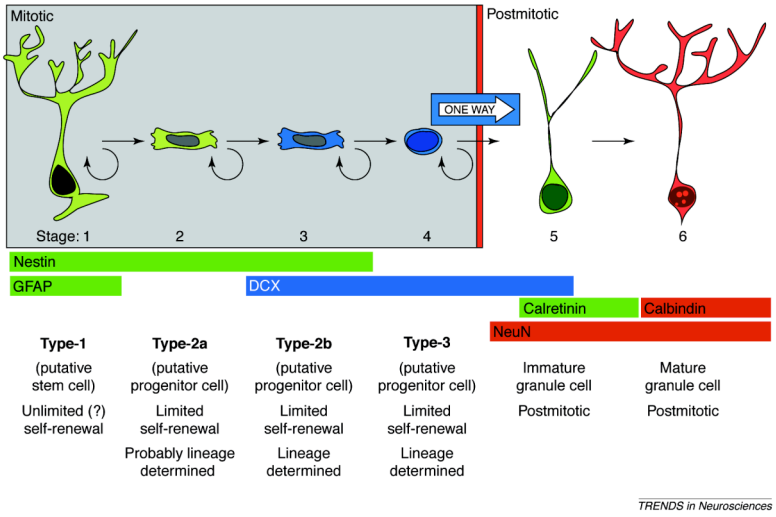


Figure 1.3: Model of adult hippocampus neurogenesis (Kempermann *et al.*, 2004).

1.3 Regulation of neurogenesis with transcription factors

To better understand the active neurogenesis taking place only in the restricted brain regions, it is important to elucidate the regulation of neurogenesis sequential phases. Adult neurogenesis is highly controlled by intracellular and intercellular factors at all steps, including proliferation, fate specification, migration, survival and synaptic

integration (Zhao *et al.*, 2008). It was identified that the genetic background effects hippocampal neurogenesis, survival and differentiation as well as total hippocampus volume in adult mice (Kempermann and Gage, 2002). Furthermore activation or repression of gene expression is controlled by transcription factors that identify gene profiles of different cell types (Hevner *et al.*, 2006). Moreover neurogenesis in the adult has similarities with the developing brain. For example, transcription factors do not only control particular steps in the differentiation of precursors into the different cell types but also regulate several aspects of neuronal identity in both adult and developing brain (Hevner *et al.*, 2006). Sets of transcription factors from different families act together to control cell fate decisions and differentiation into each lineage (Lefebvre *et al.*, 2007). However, it is really less known about transcriptional regulation of adult neurogenesis (Hodge *et al.*, 2008). Sry related high-mobility-group box (Sox) transcription factor 11 is focused on in this study to understand its role in adult neurogenesis.

1.3.1 Sry-related high-mobility-group (HMG) box (Sox) transcription factors

There are 20 identified members of Sry-related high-mobility-group (HMG) box (Sox) group proteins that are expressed in the vertebrates (Wegner, 2005). Sex-determining region on the Y chromosome Sry was the first identified Sox group family protein (Gubbay *et al.*, 1990). At the same time it was demonstrated that Sry belongs to a wide family afterwards called Sry related group box (Sox) genes (Denny *et al.*, 1992). Sox proteins regulate the transcription with its high-mobility-group (HMG) box domain. HMG box domains are associated in DNA binding, DNA bending, protein-protein interactions and nuclear import or export. When non-histone chromosomal proteins were separated by SDS-PAGE, three unrelated DNA binding proteins were identified forming a high mobility group protein. Sox group proteins are one of the groups containing HMG box domain that distinguishes in the superfamily by sharing 46% or more identity to Sry in the HMG box (Dy *et al.*, 2008). Sox group proteins bind to DNA with a specific sequence from the minor groove with the twisted L shape HMG domain included three alpha helices and an amino terminal beta strand and bend it to an angle from 30°C to 110°C (Fig 1.4). By the modification of DNA confirmation, transcription of specific genes is facilitated (Lefebvre *et al.*, 2007).

All Sox proteins bind DNA through a consensus (A/T)(A/T)CAA(A/T)G sequence but, the specificity of binding is regulated by other cell-type specific binding partners that are not clearly identified for all Sox proteins. Class III Pit-Oct-Unc (POU) transcription factors Octs and Brns have been identified as regulator binding partners of Sox proteins (Kim *et al.*, 2008).

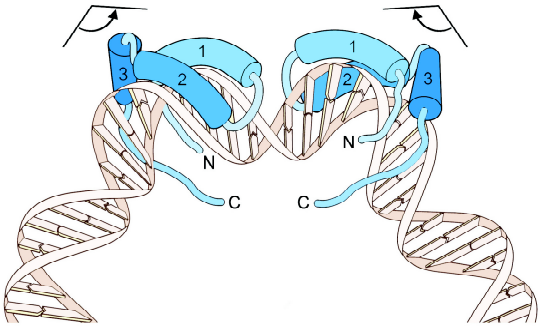


Figure 1.4: Structural properties of Sox HMG box domain. L shaped HMG box domain with 3 alpha helices (blue) binds to minor groove of linear DNA and bend it to an angle from 30°C to 110°C (Lefebvre *et al.*, 2007).

There are 8 different groups in Sox family proteins and members of the same groups share a high degree of identity (70 to 95%) within and outside of the HMG box (Table 1.1).

Table 1.1: Classification of mammalian Sox group proteins. Sox 12 and 22 are the same in mouse and in human. Sox 15 and 20 are also the same. (Wegner, 2005)

Group A	**Sry**
Group B1 B2	Sox1 Sox2 Sox3 Sox 14 Sox 21 Sox 21
Group C	Sox 4 Sox 11 Sox 12 (Sox22)
Group D	Sox 5 Sox 6 Sox 13
Group E	Sox 8 Sox 9 Sox 10
Group F	Sox 7 Sox 17 Sox 18
Group G	Sox 15 (Sox 20)
Group H	Sox 30

Sox proteins have important roles in preserving stem cell characteristics, maintaining a pluripotent state under certain conditions and cell fate determination into specific lineages as well (Wegner, 2005). For instance, specifically Sox2 acts cell autonomously to maintain the pluripotency of stem cells that later generate all embryonic and trophoblast cell types (Avilion *et al.*, 2003). Sox proteins are expressed in specific tissues during development and differentiation; therefore, mutations in these genes cause multiple developmental malformations i.e. Sox 3 mutations result in mental retardation (Laumonnier *et al.*, 2002), Sox 9 mutations are the reason of campomelic dysplesia with sex reversal (Foster et al, 1994), Sox10 mutations cause the neurocristopathy syndromes Waardenburg-Hirschsprung and Yemenite deaf-blind hypopigmentation (Inoue *et al.*, 2003). The Sox B1 genes Sox1, Sox2 and Sox3 are expressed in all neurons and have redundant roles in supplying the wide developmental potential and the identity of stem cells both in the embryo and in the adult (Wegner and Stolt, 2005). When the proneural proteins activate the expression of Sox B2 genes that repress the activity of Sox B1 proteins, development of neurons is initiated in the embryo. Additionally proneural genes activate Sox C genes (Sox11 and Sox4) that establish neuronal properties redundantly (Lfebvre *et al.*, 2007).

1.3.2 Sox11

Sox11 is a group C protein that shares a high degree identity with Sox4 and Sox22 in both HMG box domain and C terminal region (Lfebvre *et al.*, 2007). However Sox11 and Sox4 have similarities in structure and function; Sox11 activates genes more strongly than Sox4 because of more stable alpha helical structure in its transactivation domain (Dy *et al.*, 2008). Sox11 has a C terminal transactivation domain that acts as an activator synergistically with the POU proteins Brn-1 and Brn-2 (Weigle *et al.*, 2005). HMG box domain is located in the N terminal third of Sox11 and an internal acidic domain of Sox11 acts as inhibitor of its DNA binding affinity in Electron Mobility Shift Assays (EMSA) (Wiebe *et al.*, 2003). Sox11 is widely expressed in embryonic branchial arches, lung, gastrointestinal tract, pancreas, spleen, kidneys, gonads, mesenchyme and additionally human and mouse fetus in the central and peripheral nervous system (Dy *et al.*, 2008). Sox11 deficient mice died at birth from heart defects and they have developmental abnormalities in arterial

outflow, lung and skeletal formation. Additionally Sox11 deficiency causes asplenia, open eyelids, cleft lip and palate (Sock *et al.*, 2004). Sox11 ubiquitous expression in CNS and peripheral nervous system supposedly shows that it involves in development and regeneration of the brain. It has been shown that Sox11 promotes neurite growth and neuron survival. In chicken embryo, it was demonstrated that Sox11 is important for neuronal differentiation (Bergsland *et al.*, 2007). It was proposed that it is essential for expression of pan-neuronal genes like beta III tubulin. Highly activation of beta III tubulin reporter constructs by Sox11 in COS cells was concluded as beta III tubulin is the only identified downstream element of Sox11 (Bergsland *et al.*, 2007). However Sox11 is an important protein for embryonic neuronal differentiation, nothing is known about its function in the adult neurogenesis.

1.4 Aim of the study

The transcriptional cascade that regulates neuronal differentiation in adult brain is largely unknown. Sry related HMG box transcription factor Sox11 has been demonstrated to actively regulate embryonic neuronal differentiation. The aim of the study was to analyze Sox11 function in adult neurogenesis. For this purpose, Sox11 expression in adult neurogenic lineages was investigated. Sox11 expressing cells were identified by colocalization of immature neuronal markers such as doublecortin and beta III tubulin.

In this study Sox11 role in adult neurogenesis was investigated with *in vitro* Sox11 gain of function analysis. Sox11 was overexpresed in cultured adult hippocampal stem/progenitor cells (AHPs) derived from adult rats by electroporation and viral transduction. Additionally effect of Sox11 as a transcription factor on promoters of immature neuronal markers doublecortin and beta III tubulin (Tuj1) was analyzed. With this experiment if Sox11 is an upstream element of immature neuronal markers (i.e. doublecortin and Tuj1) was analyzed. Finally, short hairpin RNAs (shRNAs) against Sox11 were produced to generate a tool for loss of function analysis. Functionality of Sox11-shRNAs was tested for further approaches. Sox11 knockdown study might give more ideas about its function in adult neurogenesis.

2. MATERIALS

2.1 Chemicals

Table 2.1: List of chemicals.

Acrylamid	Sigma-Aldrich	USA
Agarose	Biozym	Germany
Ampicillin	Sigma-Aldrich	USA
Ampuwa water	Fresenius	Germany
APS	Sigma-Aldrich	USA
BSA	Sigma-Aldrich	USA
Chloroform	Roth	Germany
ECL Western blotting	Amersham	UK
D-Glucose	Sigma-Aldrich	USA
Dapi	Sigma-Aldrich	USA
Developer A	AGFA	Belgium
Developer B	AGFA	Belgium
DMEM HAM´s F12	PromoCell	Germany
DMEM(1X) with sodium pyruvate	GIBCO/Invitrogen	USA
DMEM/F-12 with GlutaMax	GIBCO/Invitrogen	USA
DMSO	Sigma-Aldrich	USA
DNA ladder (100bp)	NEB	Germany
DNA ladder (1kb)	NEB	Germany
DTT	Fermentas	Germany
ECL Hyperfilm	Amersham	UK
EDTA	Sigma-Aldrich	USA
EGTA	Roth	Germany
Ethidiumbromid (1 mg/mL)	Roth	Germany
Ethanol (100%)	Merck	Germany
Fetal Bovine Serum (FBS)	PAA	Germany
FGF2	Peprotech	USA
FSK	Sigma-Aldrich	USA
Glycerol	Sigma-Aldrich	USA
Glycin	Roth	Germany
HCl (32%)	Merck	Germany
Hepes	Roth	Germany
Isopropanol (100%)	Merck	Germany
KCl	Merck	Germany
Natural mouse laminin	Invitrogen	USA
L-Glutamine (200mM)	GIBCO/Invitrogen	USA
Loading dye (6x)	MBI Fermentas	Germany
2-Mercaptoethanol	Sigma-Aldrich	USA
Methanol (100%)	Merck	Germany
N2-supplement (100X)	Invitrogen	Germany
Normal donkey serum	Chemicon	Germany
PAGE-Prestained Protein Ruler	Fermentas	Germany
Paraformaldehyde	Roth	Germany
Passive Lysis Buffer	Promega	USA
Polymount	Polysciences	USA
PSF (penicillin, streptomycin, fungizone)	GIBCO/Invitrogen	USA
PVDF membrane	Pall Corporation	USA
Rapid fixer	AGFA	Belgium
Skim milk powder	Fluka	Switzerland
SYBR® GreenI	Invitrogen	USA
TEMED	Sigma-Aldrich	USA
Triton X-100	Roth	Germany
Trizol	Invitrogen	USA
Trizma-Base	Sigma-Aldrich	USA
Trypsin-EDTA	GIBCO/Invitrogen	USA
Tween 20	Roth	Germany

2.2 Enzymes and enzyme buffers

The enzymes and enzyme buffers used in this study are listed.

Table 2.2: List of enzyme and enzyme buffers.

HpaI	NEB	Germany
HindIII	Fermentas	Germany
NotI	NEB	Germany
RQ1 DNase	Promega	USA
SalI	Fermentas	Germany
SmaI	Fermentas	Germany
T4-Ligase	NEB	Germany
XbaI	NEB	Germany
Xho I	NEB	Germany
Enzyme buffers		
Buffer R	Fermentas	Germany
Buffer Tango	Fermentas	Germany
Ligase Buffer	NEB	UK
NEB 1/2/3	NEB	UK
PCR mix (2.5x)	Eppendorf	Germany
RQ1 Buffer	Promega	USA
RQ1 Stop buffer	Promega	USA

2.3 Kits

The kits used in this study are listed.

Table 2.3: List of kits.

Kits	**Company**	**Country**
Dual-Luciferase Reporter 1000 Assay System	Promega	USA
NucleoSpin Plasmid Kit	Macherey Nagel	Germany
QIAquick Gel Extraction Kit	Qiagen	Germany
Rat NSC Nucleofector	Amaxa Biosystems	Germany
Pure Yield Plasmid Midiprepsystem	Promega	USA
SuperScript III	Invitrogen	USA
SV Total RNA Isolation System	Promega	USA

2.4 Plasmids

The plasmids used in this study are listed.

Table 2.4: List of plasmids.

Plasmids	**Company/Suppliers**	**Country**
pLentiLox 3.7	Van Parijs Laboratory	USA
psiCHECK2	Promega	Germany
psiCHECK3	Dr. Ralf Kühn, Helmholz Zentrum,	Germany
pGL3-Basic	Promega	Germany
pKSPS	Modified pBluescript in Fred Gage	USA
pCAT-490F	Dennis *et al.*, 2006	USA
pCAT-131F	Dennis *et al.*, 2006	USA
pCAT-131R	Dennis *et al.*, 2006	USA
phuDCX3509-FFluci	Karl *et al.*, 2005	Germany
CAG-IRES-GFP	Gage Laboratory	USA
CAG-Sox11-IRES-GFP	Hisato Kondoh Laboratory	Japan
CAG-Sox11-Flag-IRES-GFP	Hisato Kondoh Laboratory	Japan

2.5 Antibodies

The antidodies used in this study are listed.

Table 2.5: List of antibodies.

Antibody	Company	Country	Catalog #	Dilution used
Primary antibodies				
chicken anti-GFP	Aves Labs, Inc.	USA	GFP-1020	1:1000
gennie pig anti-GFAP	Adv. Immuno.	USA	031223	1:1000
goat anti-doublecortin	Santa Cruz Biotechnology	USA	sc-8066	1:1000
goat anti-GFP	Molecular Probes	Germany	A-6455	1:1000
goat anti-SOX11	Santa Cruz Biotechnology	USA	sc-17347	1:500
mouse anti-ß actin	Abcam	UK	ab6276	1:1000
mouse anti-ß-III-tubulin	Sigma-Aldrich	USA	T5076	1:3000
mouse anti-calbindin	Swant	Switzerland	300	1:2000
mouse anti-flag	Sigma	USA	F1804	1:1000
rabbit anti-GFAP	DAKO cytomation	Denmark	Z0334	1:1000
rabbit anti-doublecortin	Abcam	UK	ab18723	1:1000
rabbit anti-calbindin	Swant	Switzerland	CB-38a	1:1000
rabbit anti-Sox2	Chemicon	USA	AB5603	1:1000
rabbit anti-Sox11	Chemicon	USA	AB5776	1:1000
rabbit anti-calretinin	Swant	Switzerland	769914	1:1000
Secondary antibody				
Alexa488 conjugated donkey	Invitrogen	USA	A11055	1:1000
goat anti-mouse HRP conjugated	Jackson ImmunoResearch	UK	115-035-003	1:10000
goat anti-rabbit HRP conjugated	Cell signaling	USA	7074	1:10000
Cy 5 conjugated donkey anti-goat	Jackson ImmunoResearch	UK	705-175-747	1:250
Cy 3 conjugated donkey anti-mouse	Jackson ImmunoResearch	UK	715-165-151	1:250
Cy 3 conjugated donkey anti-rabbit	Jackson ImmunoResearch	UK	711-165-152	1:250
FITC conjugated donkey anti-chicken	Jackson ImmunoResearch	UK	703-095-155	1:250
Cy 3 conjugated donkey anti-goat	Jackson ImmunoResearch	UK	705-165-147	1:250

2.6 Other equipments

The laboratory eqiupments used in this study is listed.

Table 2.6: List of other equipments.

Equipments	Company	Country
5100 Cryo 1°C Freezing container	NalgeneLabware	USA
Accu-jet pro Pipetaid	Brand	USA
BioPhotometer	Eppendorf	Germany
Blaubrand counting chamber	Brand	USA
Centrifuge 5415 D	Eppendorf	Germany
Centrifuge 5417 R	Eppendorf	Germany
Cellstar pipettes	Greiner bio-one	Germany
Confocal microscope Fluoview 1000	Olympus	USA
Cover slips	Menzel-Glaser	Germany
Cryoblock	Medite Medizintechnik GmbH	Germany
Cryotube vials	Nunc	Denmark
Curix 60 (photo developer)	AGFA	Belgium
Disposal cuvette UVette	Eppendorf	Germany
Elka microscope slides	Assistent	Germany
Fluorescence microscope DMI 6000B	Leica	Germany
Gelsystem Mini	Peqlab	Germany
Glass Cover Slips	Menzel-Glaser	Germany
HeraCell 150 incubator	Kendro	UK
HeraCell Tissue Culture hood	Kendro	UK
Light microscope	Zeiss	USA
Mastercycler ep gradient	Eppendorf	Germany
Motor pestle	Sigma Aldrich	USA
Power supply	BioRad	USA
Reaction tube 15 ml	Falcon	USA
Reaction tube 50 ml	Falcon	USA
Rotamax 150	Heidolph	Germany
Rotilabo 96 well Micro testplates and lids	Roth	Germany
Safe lock tube 1.5 ml	Eppendorf	Germany
Safe lock tube 2 ml	Eppendorf	Germany
SM2000R sliding microtome	Leica	Germany
Sorvall Evolution High Speed Centrifuge	Thermo Science	USA
SuperFrost microscope slides	Menzel-Glaser	Germany
Surgical disposal scapels	Braun	USA
Thermomixer comfort	Eppendorf	Germany
Tissue Culture dishes10 cm	Falcon	USA
Tissue Culture ware 24 well plate	Falcon	USA
Tissue Culture ware 12 well plate	Falcon	USA
Vac-man Laboratory vacuum manifold	Promega	Germany
UV spectrometer	Eppendorf	Germany

2.7 Organisms and cell lines

The used organisms and cell lines are listed.

Table 2.7: List of organisms and cell lines.

Organisms and cell lines	Company/Suppliers
E. coli Top10	F- *mcr*AD(*mrr-hsd*RMS-*mcr*BC) F80*lac*ZDM15 D*lac*X74 *rec*A1 *ara*D139 D(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G Company: Invitrogen
Human embryonic kidney (HEK) 293T cells	HEK 293 (human embryonic kidney) was first established in
Rat adult hippocampal progenitor (AHP) cells	Fred Gage Laboratory
Mouse strain C57/Bl6 male	All mice were raised in the local animal husbandry of the

2.8 Buffers and solutions

The buffers and solutions used in this study are listed together with their recepies.

Agar plates

32 g/l LB (Luria-Bertani) Agar
0.1 mg/ml Ampicillin

Agarose gel (2%)

6g	Agarose
300 ml	TAE buffer (1x)

Agarose gel (1%)

3g	Agarose
300 ml	TAE buffer (1x)

Blocking solution for immunostaining of tissue

3% donkey serum
2.5% TritonX-100
in TBS

Blocking solution for immonocytochemistry

1% donkey serum
0.1% TritonX-100
in TBS

Borate Buffer (0.1 M)

3.0915 g	Boric acid
500 ml	H_2O

pH 8.5

Cryoprotectant

250 ml	Glycin
250 ml	Ethylene Glycol
500 ml	0.1 M Phosphate buffer

HBS (2x)

8 g	NaCl
0.37 g	KCl
201 mg	$Na_2HPO_4 \cdot 7H_2O$
1 g	Glucose
5 g	Hepes

add H_2O up to 500 ml pH 7.04

Loading dye for Agarose-Gels (6x)

0.2 ml	10mM Tris/HCl pH 7.5
10 ml	Glycerol (100%)
4 ml	EDTA (0.5 M)
0.05 g	Xylencyanol
0.05 g	Bromphenol blue
20 ml	H_2O

Paraformaldehyde (4%)

40 g	Paraformaldehyde
500 ml	H_2O
Add NaOH and heat until solution is clear	
500 ml	0.2 M Phosphate buffer
pH 7.4	

PBS (10x)

80 g	NaCl
2 g	KCl
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4
800 ml	H_2O
pH 7.2	

Phosphate-Buffer (0.2 M)

16.56 g	Sodium phosphate (Na_3PO_4) monobasic
65.70 g	Sodium phosphate (Na_3PO_4) dibasic
add H_2O up to 3 L	

SDS (Sodium dodecyl sulfate) (10x)

100g	SDS
900 ml	H_2O
Heat it up to 68°C	
pH 7.2	
add H_2O up to 1 L	

SDS-Probe buffer (Laemmli-Buffer) (5x)

7.815 ml	Tris/ HCl (2 M, pH 6.8)
25 ml	Glycerin
5 g	SDS
12.5 ml	Mercapto ethanol
25 mg	Bromophenol blue
add H_2O up to 50 ml	

Sucrose (30%)

150 g	Sucrose
500 ml	Phosphate buffer (0.1 M)
Store at 4°C	

TAE (50x)

242 g	Tris Base
57.1 ml	Acetic acid
100 ml	EDTA (0.5 M, pH 8.0)
add H_2O up to 1 L	

Tris buffered Saline (TBS) (10x)

80 g	NaCl
2 g	KCl
250 ml	Tris/HCl pH 7.5 (1 M)
add H_2O up to 1 L	

TBST (10x)

1 L	TBS (10x)
10 ml	Tween 20

Western blot running buffer (10x)

30.285 g	Tris Base
144.13 g	Glycin
10 g	SDS
add H_2O up to 1 L	

Western blot transfer buffer (10x)

81.6 g	Bicine
104.6 g	Tris Base
40 ml	EDTA (0.5 M, pH 8.0)
177.5 mg	Chlorbutanol (1,1,1-Trichlor-2-methyl-2-propanol)
add H_2O up to 2 L	

Western blot transfer buffer (1x)

25 ml	Transfer Buffer (10x)
25 ml	Methanol
200 ml	H_2O

Western blot stripping buffer

3.125 ml	Tris / HCl (1 M, pH 6.8)
10 ml	SDS (10%)
add H_2O up to 50 ml	
346.6 µl	Mercaptoethanol

SDS-Polyacrylamid gel

The components of SDS-Polyacrilamid are listed.

Table 2.8: Components of SDS-Polyacrylamid gel.

Seperating gel	**10%**
1.8 ml	H_2O
1.9 ml	Lower Tris (1M pH 8.8)
1.25 ml	40% Acrylamid
50 µl	10% SDS
5 µl	TEMED
25 µl	10% APS
Stacking gel	
2.27 ml	H_2O
0.375 µl	Upper Tris (1M pH 6.8)
0.3 ml	40% Acrylamid
20 µl	10% SDS
3 µl	TEMED
30 µl	10% APS

2.9 Software

The used software is listed.

Table 2.9: Used software list.

Software		
FV10-ASW 1.6 Viewer	Olympus	USA
LAS AF Lite Leica software	Leica	Germany
Photoshop® CS3	Adobe®	USA

3. METHODS

3.1 DNA

3.1.1 Plasmid DNA isolation

Plasmid DNAs can be extracted from the bacteria by selective alkaline denaturing of chromosomal DNA as described by Birnboim and Doly (1979). After neutralization, alkaline denatured high molecular weight chromosomal DNA renatures selectively, and forms an insoluble aggregate leaving plasmid DNA in the supernatant. Plasmid DNA purification Kit (Nucleospin, Macherey-Nagel) was used for isolation of plasmids, and up to 25 µg DNA was isolated. Transfection requires pure DNA samples; thus Pure Yield Plasmid Midiprep system (Promega, #A2495, USA) was used for DNA samples that were used for transfection. All the procedures were carried according to the manufactures protocols.

3.1.2 Determination of DNA and RNA concentration

Both DNA and RNA absorb UV light very efficiently that serves concentration measurement possibility by an UV spectrometer.

DNA and RNA concentrations were determined by a photometer (BioPhotometer, Eppendorf). Absorption of the DNA sample at A_{260} nm was measured and DNA concentration was calculated with the following formulas.

1 A_{260} =50 ng/ml dsDNA

1 A_{260}=40 ng/ml RNA

3.1.3 DNA precipitation

To remove ethanol residues from DNA samples and to make dilutions for appropriate DNA concentrations additional DNA purification procedure was performed. 3 M NaAc was added in 1/10 volume of total DNA volume. Addition of 100% ethanol in 2.5x of the total DNA volume was followed by incubation on ice for 30 minutes. DNA was precipitated by centrifugation (4°C; 30 minutes; 13000 rpm) and additional wash was performed in 70% ethanol by centrifugation (4°C; 10 minutes; 13000 rpm). DNA was dried and then resolved in nuclease free water.

3.1.4 DNA fragments separation with agarose gel electrophoresis

Agarose gel electrophoresis allows separation of DNA fragments by their charge and mass differences. DNA is negatively charged because of its phosphate backbone and it migrates toward the anode when it is placed in an electric field. Furthermore, frictional force of the agarose acts as a molecular sieve for fragments that are going to be separated according to their sizes. Depending on the concentration of agarose, 0.02-50 kb long DNA fragments can be separated. Agarose gel concentration between 0.6-2% is suitable for separation of linear DNA fragments of 0.1-25 kb (Sambrook *et al.*, 1989).

DNA samples that were mixed with 6x loading buffer were loaded on a 1-2% gel that was prepared in 1xTAE buffer with 0.2 µg/ml ethidium bromide. To detect small amount of DNA samples on the gel, 2xTAE buffer was prepared with SYBR green (1:10000). DNA fragments were separated using an electrophoresis chamber (Bio-Rad) for ~1 hour at 80 mV and visualized under UV light.

3.1.5 Subcloning of DNA fragments

To be able to use a luciferase reporter plasmid, 490F, 131F and 131R Tuj1 partial promoter sequences were cut out of the pCAT basic vector (Dennis *et al.*, 2002) and cloned into pGL3 basic vector (Figure 3.1).

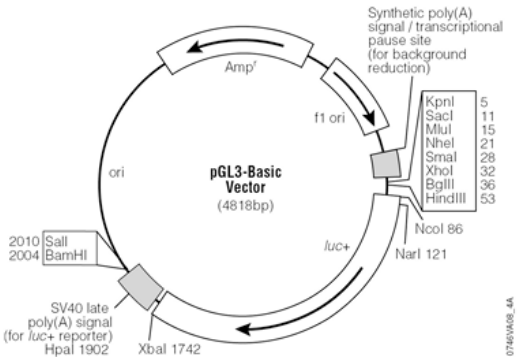


Figure 3.1: pGL3 vector map (Promega, USA).

pGL3 vector itself does not have any suitable restriction enzyme site to provide ligation of digested fragments in 5' to 3' direction; thus, after digestion from pCAT vector, pKSPS shuttle vector was used (modified pBluescript, Fred Gage laboratory, vector map is given in Appendix A). pKSPS was digested by using HindIII and SalI restriction enzymes according to the following procedure and incubated at 37°C overnight (Table 3.1).

Table 3.1: Restriction digestion reaction of pKSPS.

5 µg	DNA
0.5 µl	HindIII
0.5 µl	SalI
3 µl	Buffer
add H_2O up to 30 µl	

The digested shuttle vector pKSPS was treated with Calf Intestine Alkaline Phosphatase (CIP) for 1 hour at 37°C to remove 5' phosphate groups from the vector to avoid its self-ligation.

Double digested pKSPS and pCAT490F, pCAT131F, pCAT131R vectors were loaded on a 1% agarose gel and digested DNA fragments were extracted from the gel with the QIAquick gel extraction kit (Qiagen, #28704, Germany) according to the manufactures protocol. Before proceeding ligation reaction, concentrations of DNA fragment 490F, 131F, 131R and pKSPS were determined roughly on an agarose gel. 490F, 131F and 131R fragments were ligated into the pKSPS vector according to the following procedure and incubated at 16°C overnight (Table 3.2). T4 DNA ligase catalyses the covalent joining of DNA fragments with the requirement of ATP. Enzyme form a phosphodiester bound between 3' hydroxyl and 5' phosphate end of dsDNA fragments.

Table 3.2: Ligation reaction of pKSPS vector with Tuj1 partial promoter sequences.

5 µl	Insert (490F/131F/131R)
2 µl	pKSPS
1 µl	T4 DNA Ligase
1 µl	T4 Buffer
add H_2O up to 10 µl	

Ligation mixture was transformed into chemocompetent Top10 *Escherichia coli* bacterial cells. The ligation reaction (10 µl) was added into 100 µl of competent cells. Cells were incubated on ice for 30 minutes and subsequently heat shocked at 42°C for 1 minute. Cells were placed on ice for 2 minutes. Following addition of 1 ml LB media into the cells, cells were incubated at 37°C for 1 hour in a thermal shaker (900 rpm). 50 µl of transformed bacteria were plated on agar plates containing 75 µg/µl ampicillin and incubated at 37°C overnight. Six different single bacterial colonies were picked and grown overnight in 5 ml LB medium containing 75 µg/µl ampicillin.

Plasmid DNAs from the putative clones were purified with NucleoSpin Plamid Kit (Macherey-Nagel, #740-588.50, Germany) and digestion confirmation was performed to test the presence of inserts. Digested DNAs were analyzed on an agarose gel. Positive clones were selected and 490F, 131F and 131R fragments were cut out of the vector by double digestion of SmaI and XhoI enzymes in order to ligate fragments into the SmaI/XhoI digested and CIP treated pGL3 vector to perform luciferase assays.

3.2 RT PCR

3.2.1 Total RNA isolation from tissue

RNA was isolated from adult mouse hippocampal tissue and olfactory bulb using Trizol reagent from Invitrogen. Hippocampal tissue and tissue from olfactory bulb were dissociated from 9 weeks old adult male C57/BL mouse immediately after they were sacrificed. 1 ml of Trizol was added to 50-100 mg of tissue in a 1.5 ml reaction tube and incubated for 5 minutes at RT. Tissue was homogenized by using a 2 ml syringe. 0.2 ml chloroform was added per 1 ml Trizol reagent and the solution was vortexed for 15 seconds. After incubation at room temperature for 3 minutes, the mixture was centrifuged at 4°C 12000 rcf for 15 minutes. The aqueous phase was

then transferred to a new tube and precipitated with 0.5 ml isopropanol. After 10 minutes room temperature incubation time, centrifugation was performed at 4°C 12000 rcf for 30 minutes. The obtained RNA pellet was washed with 75% ethanol and centrifuged again (4°C; 12000 rcf; 5 minutes). The pellet was briefly dried at room temperature and dissolved in 10 µl RNAse free water. RNA concentration was measured by a photometer (BioPhotometer, Eppendorf) as described before (3.1.2 Determination of DNA and RNA concentration). Isolated RNA was stored at -80°C.

3.2.2 DNase treatment of isolated RNA

In order to remove any DNA from the isolated RNA, DNase treatment was performed with the chemicals from SV Total RNA Isolation System kit (Promega, #Z3101, USA) in the following procedure (Table 3.3).

Table 3.3: DNase treatment reaction of isolated RNA.

5 µg	RNA
1 µl	RQ DNase
1 µl	RQ DNase Buffer
add RNase free H_2O up to 10 µl	

Reaction mixture was incubated at 37°C for 30 minutes. Afterwards the reaction was stopped by adding 1µl of RQ Stop solution and incubating at 65°C for 10 minutes.

3.2.3 Complementary DNA (cDNA) synthesis

DNase treated RNA was reverse transcribed into cDNA using SuperScript III first strand cDNA kit (Invitrogen, #18080-400, USA). 5 µg of RNA was used as template for reverse transcription (Table 3.4).

Table 3.4: First strand cDNA synthesis reaction.

5 µg	RNA
1 µl	Oligo dT primer
1 µl	Annealing Buffer
add H_2O up to 8 µl	

The reaction was incubated for 5 minutes at 65°C and then immediately placed on ice for at least 1 minute. After a brief centrifugation, 10 µl 2x First-Strand reaction mix and 2 µl Superscript Enzyme mix were added on ice. After a brief centrifugation of the samples, cDNA synthesis was performed with the following program (Table 3.5) on a thermal cycler (Mastercycler ep gradient, Eppendorf). cDNA probes were stored at -20°C.

Table 3.5: First strand cDNA synthesis program.

25°C	10 minutes
50°C	50 minutes
85°C	5 minutes
4°C	∞

3.2.4 PCR

Nucleic acid sequences are enzymatically amplified via repeated cycles of denaturation, oligonucleotide annealing, and DNA polymerase extension by polymerase chain reaction (PCR) (Gibbs *et al.*, 1990). PCR amplification was performed with the cDNA from mouse hippocampus and olfactory bulb using a thermal cycler (Mastercycler ep gradient, Eppendorf).

Primers ordered from Sigma-Genosys to amplify the mouse Sox11 cDNA were used (Table 3.6). The expected size of amplified DNA is 489 bp.

Table 3.6: Primer sequences used for Sox11 amplification.

Primer	**Direction**	**Sequence (5´→ 3´)**
Mouse Sox11	Forward	TCA GCT GCT GAG GCG CTA CAG
	Reverse	GAA CAC CAG GTC GGA GAA GTT CG

PCR amplification reactions were prepared in a 25 µl reaction mixture:

Table 3.7: PCR amplification reaction.

1 µl	cDNA
0.1 µg	Forward primer
0.1 µg	Reverse primer
10 µl	2,5xPCR mix (contains Taq Polymerase, dNTP)
add H_2O up to 25 µl	

The following PCR program was used (Table 3.8).

Table 3.8: The PCR program was used to amplify Sox11.

Step	Temperature	Duration	# of Cycles
Denaturation	94°C	3 minutes	1
Amplification	94°C	15 sec	40
	68°C	45 sec	
	72°C	1 minute	
Final Extention	72°C	2 minutes	1
	4°C	∞	

PCR products were afterwards analyzed on a 2% agarose gel.

3.3 RNA Interference

RNAi that was first discovered in plants (Akashi *et al.*, 2001), Caenorhabditis elegans (Kamath *et al.*, 2001) and Drosophila (Kennerdell *et al.*, 2000) is a post-transcriptional gene silencing mechanism to protect organisms against viruses and foreign RNA molecules. When dsRNAs are introduced into a cell, ribonuclease III (RNase III) type protein Dicer processed them to ~22 nt short interfering RNA (siRNA) duplex with 2-nt overhangs at each 3' end (Bernstein *et al.*, 2001). Generated siRNAs are associated with the RNA-induced silencing complex (RISC) that specifically targets and cleaves homologous RNA sequences (Bernstein *et al.*, 2001). After introducing chemically synthesized siRNAs mimicking Dicer cleaved substrates to an organism, they specifically silence gene of interest (Elbashir *et al.*, 2001). To avoid transient effect of siRNA knockdown, DNA based expressed siRNAs called short hairpin RNAs (shRNA) are generated (Kim, 2003). shRNAs are cloned into plasmid after a RNA polymerase III promoter such as U6. When they are transcribed by RNA polymerase III in the nucleus, Dicer cleaves them to generate siRNAs (Kim, 2003).

To knock down Sox11, 4 different shRNA constructs were designed with a length of 19 bases. They were designed with siRNA Target Designer program from Promega (www.promega.com/siRNADesigner/default.htm) and one of the siRNA target sequences was the same used by Bergsland *et al.* (2007). For primer designing, T was added to the beginning of the target sequence, G(N_{18}), to reproduce U6 promotor in -1. A loop sequence (TTCAAGAGA) was added behind the G(N_{18}) that was then followed by reverse complement of G(N_{18}). 6 Ts were added as a terminator sequence (Figure 3.2). The antisense primer was designed with additional 5′ end AGCT nucleotides to produce XhoI overhang. Primers were synthesized by Sigma-Genosys (Table 3.9).

loop sequence XhoI overhang

5' T(sense) TTCAAGAGA (antisense) TTTTTTC 3'

3' A(antisense)AAGTTCTCT (sense)AAAAAAGAGCT 5'

Figure 3.2: Representative figure of Sox11-shRNA primers.

Table 3.9: Sox11-shRNA primer sequences.

Primers	Direction	Sequence (5´→ 3´)
Sox11-shRNA 1	Forward	TGGCGTCGGGCCACATCAAATTCAAGAGATTTGAT GTGGCCCGACGCCTTTTTTC
	Reverse	TCGAGAAAAAAGGCGTCGGGCCACATCAAATCTCT TGAATTTGATGTGGCCCGACGCCA
Sox11-shRNA 2	Forward	TGCCTTCATGGTGTGGTCCATTCAAGAGATGGACC ACACCATGAAGGCTTTTTTC
	Reverse	TCGAGAAAAAAGCCTTCATGGTGTGGTCCATCTCT TGAATGGACCACACCATGAAGGCA
Sox11-shRNA 3	Forward	TGAGAAGATCCCGTTCATCATTCAAGAGATGATGA ACGGGATCTTCTCTTTTTTC
	Reverse	TCGAGAAAAAAGAGAAGATCCCGTTCATCATCTCT TGAATGATGAACGGGATCTTCTCA
Sox11-shRNA 4	Forward	TGGCGGCCGGCTCTACTACATTCAAGAGATGTAGT AGAGCCGGCCGCCTTTTTTC
	Reverse	TCGAGAAAAAAGGCGGCCGGCTCTACTACATCTCT TGAATGTAGTAGAGCCGGCCGCCA

Two complementary Sox11 primers were annealed in the annealing buffer (100 nM Tris-HCl pH 7.5, 1 M NaCl, 10 mM EDTA, DEPC-treated H_2O) at 65°C for 10 minutes (Table 3.10).

Table 3.10: Oligoligation reaction of Sox11-shRNA primers.

Primer forward (100 pM)	10 µl
Primer reverse (100 pM)	10 µl
Annealing buffer	5 µl
add H_2O to 50 µl	

Then, the reaction mixture was slowly cooled down to room temperature for 2 hours. Annealed shRNA primers that have one blunt end and one XhoI overhang were cloned into HpaI/XhoI digested pLentiLox 3.7 vector (Figure 3.3). In this study lentiviral system was used to deliver shRNAs both into dividing and nondividing cells. Additionally lentivirally delivered transgenes are not silenced in developmental phases that can be used to produce transgenic animals. It was shown that pLentiLox 3.7 lentiviral vector delivered shRNAs silenced gene expression very stable and specific in different cell populations (Rubinson *et al.*, 2003).

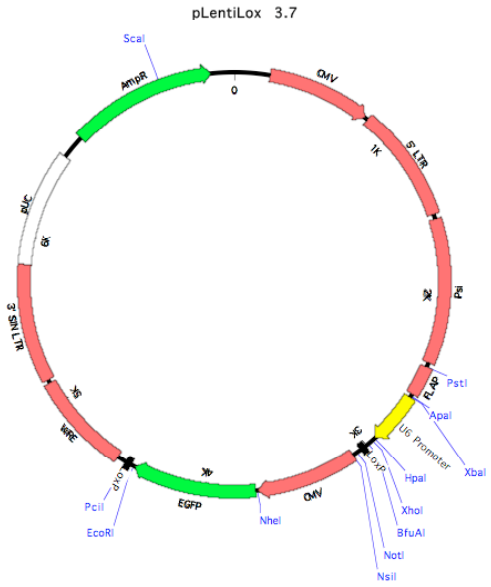


Figure 3.3: pLentiLox 3.7 vector map (Van Parijs Laboratory, USA)

3.4 Cell Culture

In this study an adult (8-10 weeks old) rat hippocampal progenitors (AHPs) cell line was used. The isolation, characterization and culturing of AHPs used in this study have been described previously (Palmer *et al.*, 1997; Ray and Gage, 2006). Additionally, HEK 293T cells, which are human renal epithelial cell line, were used to be expressed cellular proteins. 293T express SV40 large T antigen that supplies episomal replication of SV40 origin and early promoter region included plasmids. All cells were cultured in a cell incubator (HeraCell 150 incubator, Kendro) at 37°C with 5% CO_2.

3.4.1 Culturing of rat AHPs

Rat AHPs were cultured in DMEM/F-12 (GIBCO 31331, +GlutaMax) media supplemented with 1×PSF and 1×N2 supplement. FGF2 (20 ng/µl) was added to the media. Cells were grown on polyornithine laminin coated tissue culture plates (Falcon, 10 cm). Media containing FGF2 was changed every 2nd day. When the plates were confluent with cells, cells were passaged by trypsinization. 1 ml trypsin was added and removed from the plate quickly. After incubation at 37°C for 2

minutes, cells were washed from the plate with 5 ml media and then pelleted in a 15 ml Falcon tube via centrifugation 2 minutes at 0.3 rcf. The pellet was resuspended in 1 ml media. 100 µl or 200 µl of diluted cellular pellet was used for 1:10 or 1:5 10 cm plate preparation. To split the cells on 24 well plates, cells were counted and plated at a density of $5x10^4$ cells per well.

3.4.2 Freezing and thawing cells

Cells that were pelleted as described before were resuspended in conditioned medium containing 10% DMSO to freeze them. Frozen cells were stored at -80°C. For thawing the cells, they were incubated at 37°C for a few minutes. 10 ml of media was added dropwise to the cells in order to slowly dilute the DMSO. Subsequently cells were pelleted at 0.3 rcf for 2 minutes. The pellet was resuspended in fresh media and cells were plated on tissue culture plates and cultured as described above.

3.4.3 Electroporation of rat AHPs

Rat AHPs were electroporated with the Rat NSC Nucleofector kit (Amaxa Biosystems) for Sox11 or as GFP protein overexpression. Expression vectors both have strong and ubiquitous CAG promoter that is a combination of the human cytomegalovirus immediate-early enhancer and a modified chicken beta-actin promoter (Figure 3.4). Sox11 is expressed as a GFP fusion by this vector.

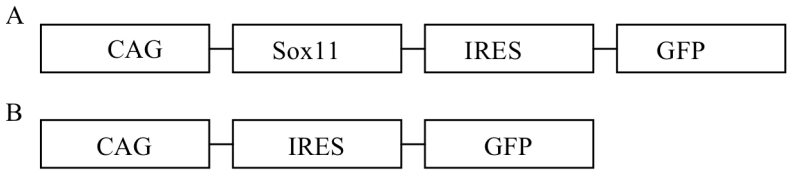


Figure 3.4: Schema of expression vectors. (A) Sox11 and (B) GFP.

A confluent 10 cm plate was taken and cells were pelleted as described before. Four different electroporations can be done with one confluent plate. The pellet was resuspended in 100 µl/electroporation rat Nucleofector solution. This mixture was added to the DNA of interest and transferred to an Amaxa certified cuvette. After electroporation was performed with the rat high efficiency A-033 program, the cuvette was rinsed with 500 µl culture medium. This solution was transferred into a 15 ml Falcon tube, followed by an addition of 2.5 ml culture medium addition. The

electroporated cells were mixed gently with media and 500 µl/well of mixture was transferred onto coated glass coverslips in a 24 well plates. The cells were then kept overnight under proliferating conditions and then the medium was exchanged and the cells were kept 3 more days under differentiation conditions.

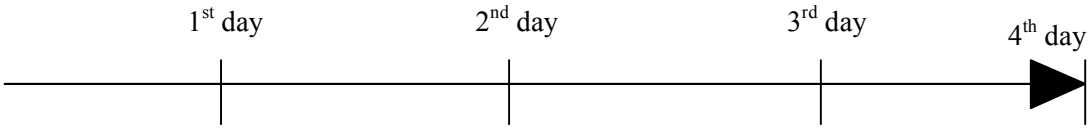
3.4.4 Viral transduction of rat AHPs

Rat AHPs were transduced with Sox11 and or GFP retroviruses. Here the same constructs that were electroporated into rat AHPs were used to prepare retroviruses. For retroviral production, 20 cm 80-90% confluent 293T cell plates were split down to 10-12 cm plates. Cells were transfected using Lipofectamine 2000 Reagent (Invitrogen). For each 10 cm plate, 9 µg of Sox11/GFP overexpression construct, 6 µg of CMVgp (promoter sequence of retrovirus) and 3 µg of vsvg (viral packaging sequence) were diluted in 1.5 ml Opti-MEM I (Invitrogen) and then combined with 60 µl Lipofectamine 2000 Reagent that was diluted in 1.5 ml Opti-MEM Reduced-Serum Medium. After 30 minutes incubation at room temperature, 3 ml of DNA-Lipofectamine 2000 complex was added to each 10 cm plate. Plates were incubated overnight at 37°C. Transfection media was changed with D-MEM including fetal calf serum (FCS) and PenStrep. Plates were incubated for 48 hours at 37°C before collecting the virus. Because produced viruses were in the cellular media, virus containing media was centrifuged in the ultracentrifuge (4°C; 50000 rcf; 90 minutes). After the whole supernatant was aspirated off, the viral pellet was resuspended in TNE (50 mM TrisHCl pH 7.8, 130 Mm NaCl, 1 mM EDTA). Different concentrations of prepared viruses were transfected into 293T cells to measure the titer. The titer of the prepared Sox11-CAG-IRES-GFP or CAG-IRES-GFP viruses were both ~$1.8x10^5$ virus particle/ml. Sox11-CAG-IRES-GFP virus or the control virus were added 1 µl/well to the cultured rat AHPs in 24 well plate with coated glass cover slips which were plated with a density of $5x10^4$ cells/well. The cells were then kept overnight under proliferating conditions and then for 3 more days under differentiation conditions (Explained in 3.4.5 Differentiation assay).

3.4.5 Differentiation assay

A neuronal differentiation assay of rat AHPs was performed. Rat AHPs were plated after electroporation (Explained in 3.4.3 Electroporation of rat AHPs) onto coated glass cover slips in 24 well plates. Cells were grown overnight in their respective

growth medium with FGF2 to keep them under proliferating conditions. On the next day the 1st group of cells was cultured in normal media without FGF2 to promote differentiation. Furthermore to enhance differentiation, forskolin (Fsk) (5µM) and fetal bovine serum albumin (FBS) (0.5%) were added to the 2nd group of cell media as described before (Palmer *et al.*, 1997). Cells were kept under these (differentiating) conditions for 3 more days (Figure 3.5).



1st condition	+FGF2	with no treatment
2nd condition	+FGF2	with Fsk(5µM) and FBS (0.5%)

Figure 3.5: Culturing conditions for neuronal differentiation of AHPs.

Afterwards, the cells were fixed with 4% PFA (pH 7.4) for 10 minutes and subsequently washed three times with TBS. Cells were stored in TBS until processed for immunocytochemistry.

3.4.6 Coating of plates

To grow rat AHPs as an adherent monolayer, Laminin and poly-Ornithin coated tissue culture plates were required. 5 ml Poly-ornithin (P-Orn) solution (10 µg/ml) was added to 10 cm plates (Falcon) to coat them. After incubation overnight under the tissue culture hood, plates were washed two times with sterile ampuwa water. Subsequently, 5 ml Laminin/PBS solution (5 µg/ml) was put on each plate for 24 h. Plates were wrapped in plastic on the next day and stored at -20°C until used.

Glass cover slips were coated in 24 well plates. Glass cover slips were washed in acetone for 20 minutes and afterwards incubated in 100% ethanol overnight on the shaker before coating. Furthermore, they were incubated in 1 M HCl for one hour on the shaker on the next day and subsequently washed several times in sterile water. Washed glass cover slips were stored in 100% ethanol until used. For coating, washed glass cover slips were put into the wells of 24 well plates and were sterilized for 30 minutes by UV-light exposure under the tissue culture hood. The coating protocol described for the 10 cm plates was used with 50 µg/ml final P-Orn

concentration. 500 µl of both P-Orn and Laminin solutions were used to cover each well with the cover slip.

3.4.7 Culturing of HEK 293T cells

293T-cells were cultured in DMEM 1x (GIBCO 41966, +4.5 g/L Glucose, L-Glutamine and Pyruvate) supplemented with 1xPSF and 10%FBS. Medium was changed every 2nd day. When the plates were confluent, cells were passaged by trypsinization. To split cells on 24 well plates, cells were counted and at a density of $3x10^4$ cells plated per well.

3.4.8 Transient transfection of HEK 293T cells via calcium phosphate method

Exponentially growing cells were replated in growth medium 24 hours before transfection and then transfected at 60-70% confluence. For transfection of 10 cm plates, maximum 10 µg DNA was mixed with H_2O in 1095 µl total volume. 155 µl 2 M $CaCl_2$ was added. Then 1250 µl of 2xHBS was added dropwise while gently mixing. The transfection mixture was incubated at room temperature for 3 minutes and then added to the growth media of the cells. After 24 hour, the medium of the transfected cells was refreshed. Cells were harvested 48 hours after transfection. For transfection of 24 well plates, half of the reaction mix described before was prepared and of this a 100 µl was added per well.

3.5 Luciferase Assay

Luciferase protein catalyzes the ATP dependent oxidation of luciferin that ends up in detectable light emission. Luciferase assay was performed not only to analyze the effect of Sox11 protein on DCX and Tuj1 promoters but also to test the knock down efficiency of the produced shRNA constructs against Sox11.

For Tuj1 promoter analysis, 1.5 µg pGL3 vector that has partial promoter sequences, 3 µg of Sox11-IRES-GFP/CAG-GFP with 0.1 µg *Renilla* luciferase vector were prepared in calcium phosphate transfection mix. For DCX promoter analysis, 1.5 µg phuDCX3509-FFluci (vector map is given in Appendix A) and Sox11-IRES-GFP in different concentrations (8 ng, 16 ng, 24 ng, 32 ng, 40 ng, 80 ng, 160 ng) with 0.1 µg *Renilla* luciferase vector were prepared in calcium phosphate transfection mix. HEK 293T cells on 24 well plates were transfected with described transfection mix and

were lysed 48 hours after transfection with 150 µl/well Passive Lysis Buffer (in 1:5 dilution with H_2O). Chemiluminescense was measured with 5 µl of cell lysate by the luminometer. The luciferase activity was calculated in Relative Light Units (RLU) and as a ratio of Firefly activity/*Renilla* activity.

shRNAs were tested quantitatively in psiCHECK^{TM}-2 vector with luciferase activity measurement. psiCHECK^{TM} vectors give the possibility to check the shRNAs effect on the expression of a target gene fused to the reporter gene. Reporter gene of the vectors is *Renilla* luciferase and the gene of interest can be cloned into downstream of *Renilla* luciferase translational stop codon. When the shRNA is produced against the gene of interest, fusion mRNA is degraded by the cleavage of shRNA (Figure 3.6). Decreased *Renilla* luciferase activity gives the shRNA effect in quantitative manner.

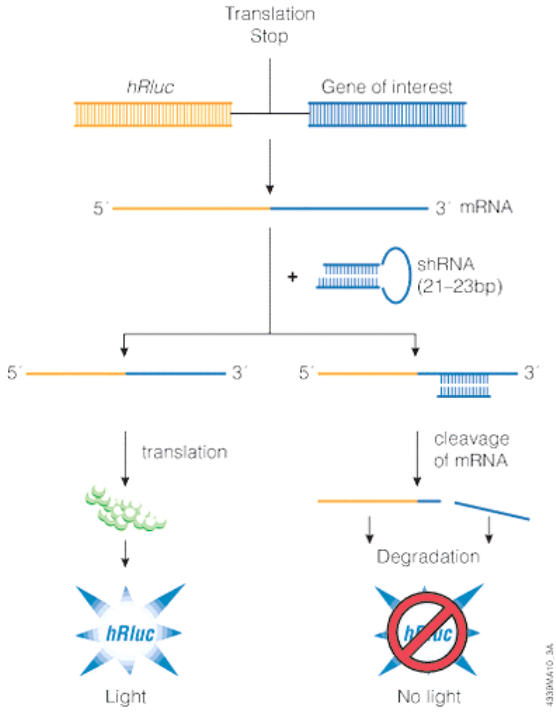


Figure 3.6: Action mechanism of psiCHECK^{TM}-2 vector.

To test the knock down efficiency of shRNAs against Sox11, full length Sox11 gene sequence was cloned into 3' of the *Renilla* luciferase translational stop codon in psiCHECK^{TM}-2 vector (Figure 3.7). The vector has additionally a firefly luciferase sequence driven by a second promoter to be used as an internal control of luciferase activity. 0.25 µg of psiCHECK^{TM}-2 vector including full length Sox11 and 0.25 µg of shRNA constructs in pLentiLox 3.7 vector were transfected into HEK 293T cells.

Cell harvesting and luciferase activity measurement were done as described before. The luciferase activity was calculated in Relative Light Units (RLU) and as a ratio of *Renilla* activity/Firefly activity.

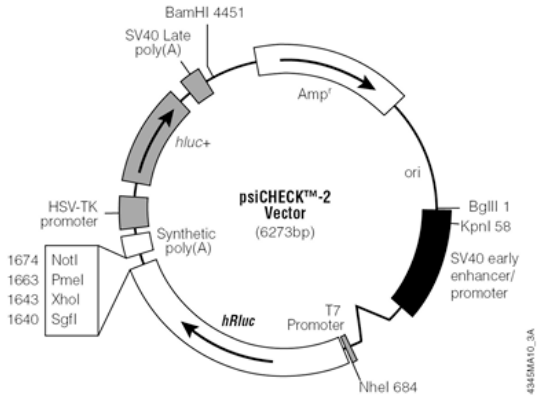


Figure 3.7: psiCHECK^{TM}-2 vector map.

3.6 Protein Biochemistry

3.6.1 Preparation of protein extracts from cells

Transiently transfected HEK 293T cells were used for protein extract preparation. The used assay is based on different salt concentrations specialized to isolate transcription factors (Bumsted-O´Brien *et al.*, 2007). Cells of confluent HEK 293T cell 10 cm culture plates (Falcon) were lysed with 150 µl Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT) placed on ice for 5 minutes and shortly vortexed after 10% 10x Igepal addition. Cellular debris was pelleted by centrifugation for 1 minute at 10000 rpm (4°C). The supernatant containing the cytoplasmic proteins was separated from the pellet, which contains the nuclear extract. The nuclear membrane was degraded after addition of 90 µl Buffer B (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 400 mM NaCl, 1% Igepal) by mixing in overhead shaker (4°C; 15 minutes). After a centrifugation at 10000 rpm for 5 minutes the supernatant, which contains the nuclear proteins, was combined with cytoplasmic proteins.

3.6.2 Protein concentration measurement

Protein concentration was determined by the Bradford Protein Assay (BioRad) with the spectrophotometer (BioPhotometer, Eppendorf). The principle of the assay is

shifting of the maximum absorbance of acidic dye Coomassie Brilliant Blue G-250 from 465 nm to 595nm when binding to a protein occurs.

Bradford assay reagent was prepared with 1:4 dilutions in H_2O. Protein standard curve was made with protein standards prepared in the same buffer combination as the samples to be assayed. According to the standard curve, protein concentration was measured.

3.6.3 Electrophoresis of proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their electrophoretic mobility. SDS is an anionic detergent that dissolves hydrophobic molecules and attached them a negative charge. When a cell is treated with SDS, the membranes will be dissolved, all the proteins will be solubilized, their secondary-tertiary structures will be denatured and they will be applied negative charge in proportion to their weight by the detergent. When the denatured proteins are applied to polyacrylamide gel submerged in a suitable buffer, an electric current is applied across the gel that causes the migration of negatively charged proteins across the gel towards the anode. Each protein will migrate differently depending on their size and they will be separated through the gel matrix.

Proteins were resolved on SDS polyacrylamide gels using the Bio-Rad vertical mini gel system. Equal amounts of proteins (100 μg) were mixed with 5x Laemmli buffer and were denatured at 95°C for 5 minutes. Denatured proteins were loaded on a 10% SDS-Polyacrylamide gel. The proteins were collected on the stacking gel at 50 V and then they were separated on separation gel at 100 V. Running buffer recipe for SDS-PAGE is described in Buffers and Solutions.

3.6.4 Western blot

Proteins were transferred from the gel to a PVDF (Polyvinylidenfluorid) membrane (Pall Corporation) with blotting buffer in a blotting apparatus from Invitrogen. Blotting was performed at 30 V for 1 hour. Afterwards the membrane was blocked for 1 hour in 5% skim milk powder in TBST and incubated with primary antibody diluted in 3% BSA added TBST over night at 4°C. The next day, the membrane was washed 3 times for 10 minutes in TBST and then incubated in HRP conjugated secondary antibody at room temperature for 1 hour. Then the membrane was again

washed one time in TBST and then three more times in TBS for 10 minutes of each washing step. The bound antibodies were detected with the ECL chemiluminescence detection kit (Amersham). The membrane was exposed to X-ray sensitive film (ECL Hyperfilm, Amersham) which was developed in the photo developer (Curix 60, AGFA). Transfer buffer recipe for blotting is described in Buffers and Solutions.

3.6.5 Stripping of Western blot membranes

The previously bound antibodies to the used membranes can be removed by stripping. This allows to reprobe the membrane by using a second set of antibodies.

The membrane was incubated in stripping buffer for 30 minutes at 37°C. After one washing step in TBST for 10 minutes the membrane can be re-blocked in 5% skim milk powder in TBST for 1 hour and afterwards incubated with primary antibody in 3% BSA added TBST. Stripping buffer recipe is described in Buffers and Solutions.

3.7 Immunohistochemistry

3.7.1 Immunostaining of brain slices

C57/Bl6 mice were deeply anesthetized and perfused transcardially with 4% PFA in PBS (pH 7.4). Brains were removed, post-fixed in 4% PFA for 24 hours and placed in 30% sucrose solution. Brains were cut both coronally and sagittally into 40 μm thick sections on a sliding microtome (SM2000R sliding microtome, Leica) on dry ice. Sections were collected and stored for further investigations in cryoprotectant solution at –20°C. Selected free floating sections for immunostaining were rinsed in TBS three times for 15 minutes in a 12-well plate with nets. Sections were incubated in blocking solution (TBS^{++}; TBS containing 3% donkey serum and 0,25% Triton-X-100) for one hour at RT. Sections were then incubated with primary antibody in TBS^{++} for 72 hours on overhead shaker at 4°C. On the forth day, sections were washed 3 times in TBS for 15 minutes and then once with TBS^{++} for 30 minutes. Afterwards sections were incubated for 1 hour with fluorescent conjugated secondary antibodies diluted in TBS^{++}. Then following one washing step in TBS, sections were washed once with TBS containing DAPI (1:10000). After the last washing in TBS, sections were mounted on slides with polymount. Then sections were investigated under the microscopy.

3.7.2 Immunostaining of cells

Fixed rat AHP cells that were stored in TBS were blocked with TBS++ (TBS containing 1% donkey serum and 0.1% Triton-X-100) for 1 hour. Afterwards cells were treated with primary antibody in TBS++ over night at 4°C. On the second day, cells were washed three times with TBS and subsequently blocked with TBS++ for 30 minutes. Cells were incubated in TBS++ containing a fluorophore coupled secondary antibody for 2 hours at room temperature. Following one washing step with TBS, cells were washed once with TBS containing DAPI (1:10000). After one further washing step with TBS, cells were ready for investigation under the microscope. If they were grown on glass cover slips, cover slips were mounted on slides with Polymount and afterwards investigation was performed.

3.7.3 Microscopy

A light microscope (Zeiss), two fluorescence microscopes (DMI 6000B Leica, Axiovert 200M Zeiss) and a confocal microscope (Fluoview 1000 from Olympus) were used.

3.7.4 Counting procedure

To determine GFP and DCX positive cells, whole coverslip area of every well was observed under 20x objective of fluorescence microscope (Fluorescence microscope DMI 6000B, Leica; Axiovert 200M Zeiss). The number of GFP positive cells was counted in order to determine the percentage of both GFP/DCX and Sox11-IRES-GFP/DCX double positive cells.

4. RESULTS

4.1 RT PCR reveals that Sox11 is expressed in neurogenic lineages

Sox11 expression was examined in adult brain/neurogenic lineages by RT PCR. For RT PCR, total RNA was extracted from the hippocampus and olfactory bulb using the Trizole reagent. cDNA was synthesized and with the first strand cDNA, PCR was performed with specific primers to amplify 489 bp sequence of Sox11. As a control, first strand cDNA reaction of isolated RNAs was done without reverse transcriptase addition (-RT) to exclude amplification of Sox11 out of genomic DNA. As a negative control the PCR was run with a sample containing H_2O instead of cDNA (-cDNA). Amplified products were run on a 2% agarose gel and visualized with SYBR green (Figure 4.1). A 489 bp product of Sox11 was detected in both regions of the adult brain tested, in the hippocampus and olfactory bulb. The negative control shows the specificity of the results.

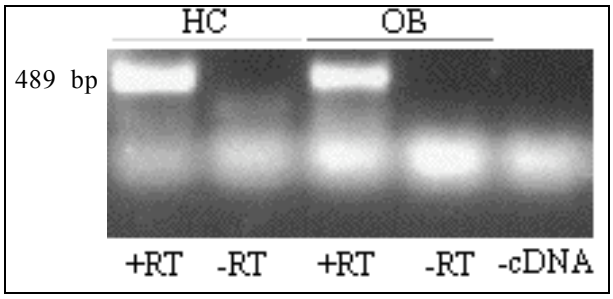


Figure 4.1: Sox11 expression in adult hippocampus and olfactory bulb. RT PCR from total RNA of hippocampus and olfactory bulb reveals Sox11 expression. +RT = Reverse transcriptase included: -RT = without reverse transcriptase: -cDNA = negative control.

4.2 Sox11 is expressed in newborn immature neurons in the adult neurogenic lineages

Anja Badde identified Sox11 expression pattern for the first time in adult neurogenic lineages with immunohistochemical detection of Sox11 in combination of different neural precursor markers and also mature neuronal markers (unpublished data). She showed that Sox11 is expressed in immature neurons in adult neurogenic lineages. In this present study, immunohistochemical staining of Sox11 was repeated to confirm these results.

Aim of this experiment was to identify the cell types that express Sox11 in the RMS/OB system or hippocampus. To reveal this, immnuhistochemical detection of Sox11 in combination with different cell markers was performed. Cryosections were immunostained with Sox11 specific antibody in combination with Sox2 or GFAP as stem cell markers and doublecortin or beta III tubulin (Tuj1) as early neuronal markers and analyzed in the RMS/OB system and the hippocampus.

As it is shown in Figure 4.2, there are Sox11 positive cells along the rostral-migratory stream but most of them are GFAP or Sox2 negative (A). Nevertheless most of the Sox11 positive cells are doublecortin and beta III tubulin (Tuj1) positive (Figure 4.2 B). As a result, migrating neuroblasts express Sox11 that migrate along the RMS towards to the olfactory bulb.

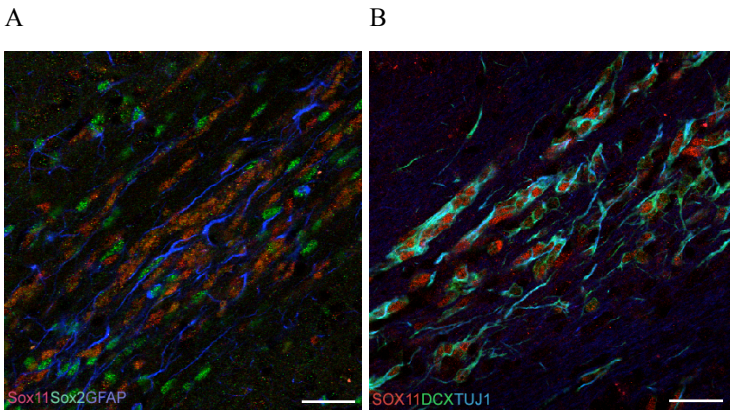


Figure 4.2: Sox11 expression in the RMS. In the RMS most of the Sox11 (red) positive cells are not Sox2 (green) or GFAP (blue) positive (A) but are doublecortin (green) and Tuj1 (blue) positive (B). Scale bar: 30 μm

Additionally, to identify Sox11 expression in the hippocampus, brain sections were stained with Sox11 specific antibody in combination with the stem cell marker Sox2 or GFAP, the early neuronal marker doublecortin, calretinin or beta III tubulin (Tuj1) and the mature neuronal marker calbindin. According to the Figure 4.3, there are Sox11 positive cells located in the dentate gyrus, which are Sox2 or GFAP negative (Figure 4.3 A), but all Sox11 positive cells are positive for doublecortin and most of them also colocolized with beta III tubulin (Tuj1) (Figure 4.3B). Additionally there is an overlap between calretinin and Sox11 positive cells, whereas there is no overlapped staining between calbindin and Sox11 (Figure 4.3C). Consequently, Sox11 is said to be expressed in dentate gyrus in immature neuronal precursors.

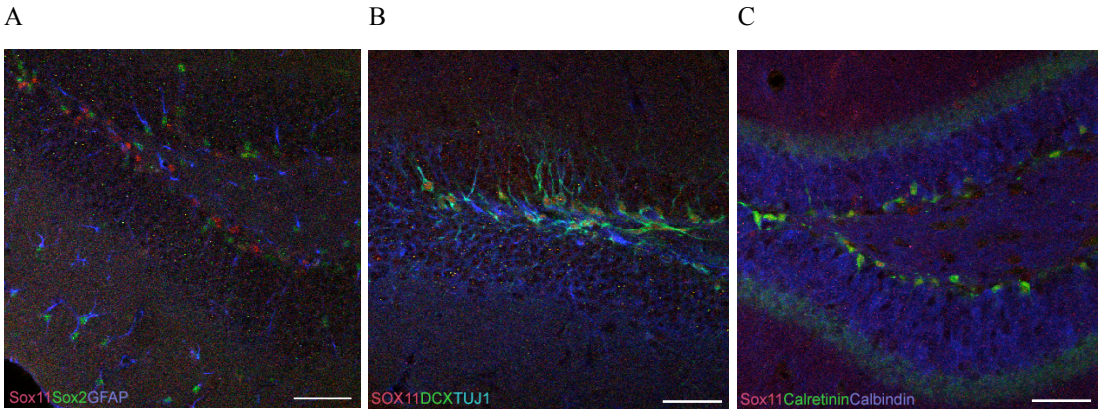


Figure 4.3: Sox11 expression in the hippocampus. A) Most of the Sox11 (red) positive cells do not express Sox2 (green) or GFAP (blue). B) Sox11 positive cells are doublecortin (green) and Tuj1 (blue) positive. C) Some Sox11 positive cells are calretinin (green) positive but all of them are calbindin (blue) negative. Scale bar: 50 μm.

A schematic drawing modified from Encinas *et al.* summarizes the expression pattern of Sox11 in the hippocampus (Figure 4.4).

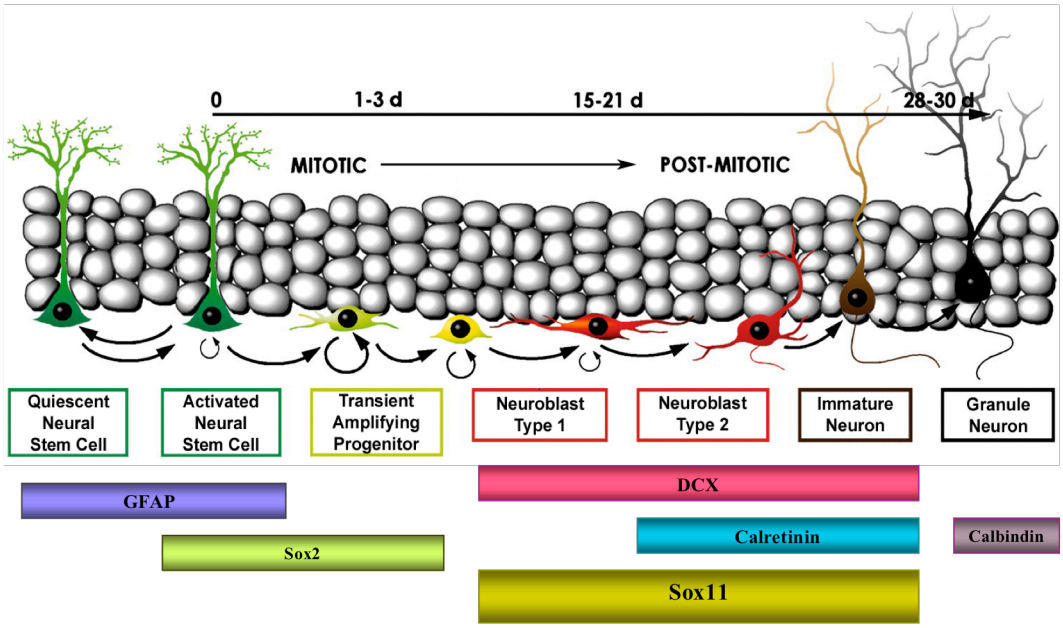


Figure 4.4: Schematic drawing of Sox11 expression in developmental stages of the adult hippocampus neurogenesis (modified from Encinas *et al.*, 2006).

4.3 Sox11 gain of function analysis

Since Sox11 expression in neurogenic lineages was detected, *in vitro* gain of function analyses of Sox11 were performed to understand its role in adult neurogenesis. According to the observed Sox11 overlapped expression with doublecortin (DCX) and Tuj1 in tissue sections, the question for *in vitro* analyses were based on the relation in between Sox11 and DCX/Tuj1.

4.3.1 Activation of the DCX promoter by Sox11

To analyze whether Sox11 is able to induce the DCX promoter, phuDCX3509-FFluci which contains the 3509 bp putative human DCX regulatory sequence driving the firefly luciferase gene (Karl *et al.*, 2005) was transiently transfected to HEK 293T cells together with Sox11-CAG-IRES-GFP vector in a dose dependent manner (8 ng, 16 ng, 24 ng, 32 ng, 40 ng, 80 ng, 160 ng). Firefly luciferase activity was normalized against the Tk driven *Renilla* luciferase. Since negative control for the transfection was CAG-IRES-GFP coding for green fluorescence protein instead of Sox11 was transfected together with phuDCX3509-FFluci. This study shows that Sox11 induces DCX promoter. Small dose (8 ng /30x10^6 cells) of Sox11 is already sufficient to 50 folds activation of DCX promoter (Figure 4.5).

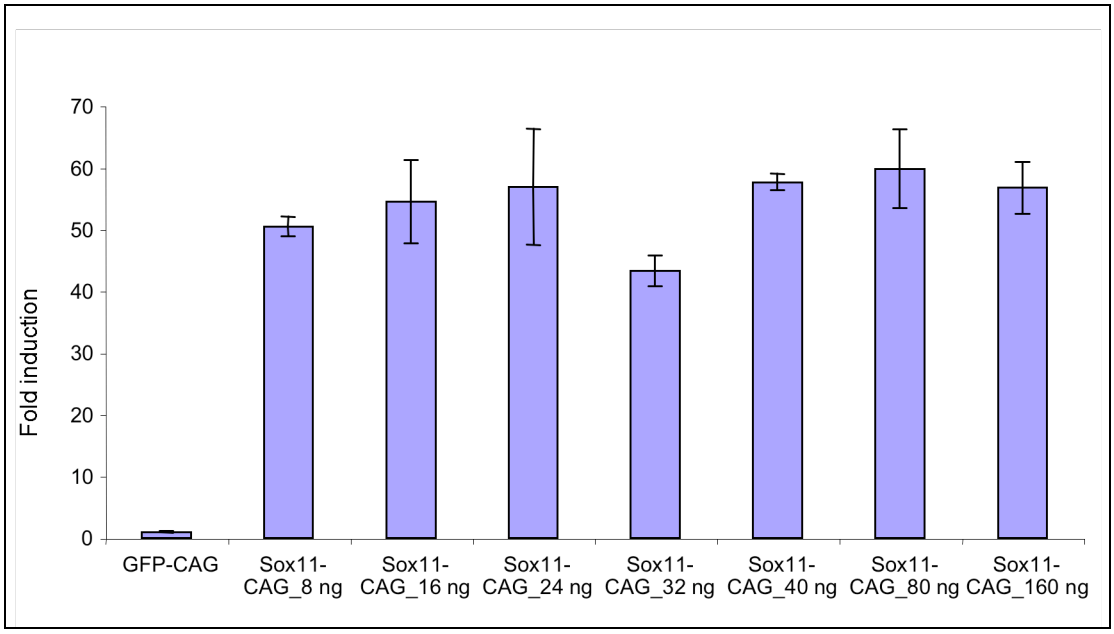


Figure 4.5: Sox11 activates DCX promoter. Transactivation of DCX-promoter reporter by Sox11 in a dose dependent manner.

4.3.2 Sox11 overexpression induces DCX expression in AHPs

Neurons, astrocytes and oligodendrocytes can be derived from adult hippocampal progenitors (AHPs) that are multipotent cells (Gage, 2002). To identify Sox11 function *in vitro*, Sox11 was overexpressed in AHPs and was kept under neuronal differentiating conditions to test for a possible influence of Sox11 on neuronal differentiation.

Sox11-CAG-IRES-GFP or CAG-IRES-GFP control vectors were electroporated into rat AHPs. Electroporated AHPs were cultured with FGF2 containing medium (+FGF2) overnight for proliferation of precursors. On the second day the medium was changed. In the first experimental condition the cells were cultured without FGF2 (-FGF2), and in the second experimental condition, the cells were treated with forskolin (5mM) and fetal bovine serum (0.5%) for 3 more days to trigger the differentiation. Exposure of AHPs to forskolin and fetal bovine serum has been shown to promote differentiation (Ray and Gage, 2006). Four days after electroporation, cells were fixed with 4% paraformaldehyde and immunostained for GFP and DCX specific antibodies. Then quantification was performed for Sox11-IRES-GFP and GFP positive cells that were double labeled with DCX (Figure 4.6).

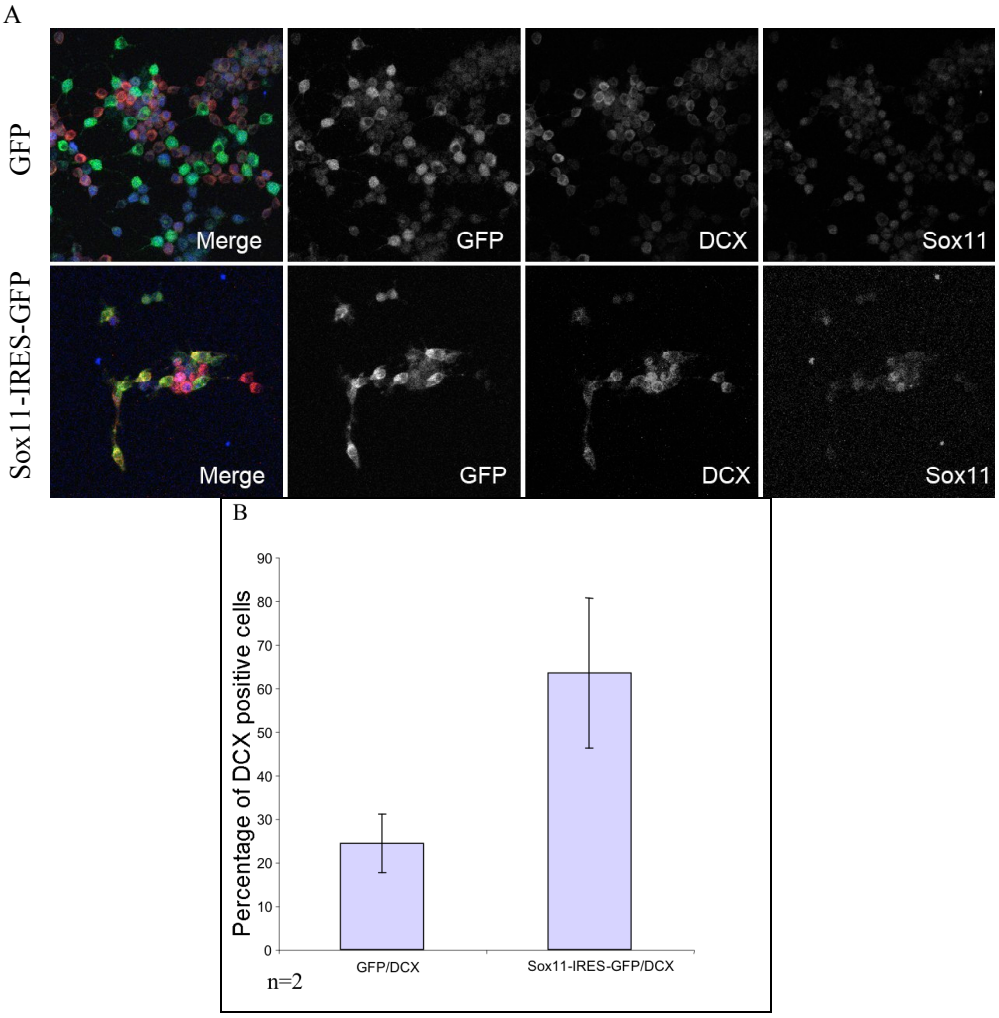


Figure 4.6: Sox11 overexpression induces DCX expression of AHPs kept under – FGF2 conditions. Electroporated AHPs were kept one day under proliferative conditions +FGF2 then three more days under neuronal differentiation conditions – FGF2. Four days after electroporation, cells were fixed and immunostained with GFP, DCX and Sox11 specific antibodies (A). A quantification of GFP/DCX and Sox11-IRES-GFP/DCX double positive cells is shown in (B). Error bars indicate standard deviation of two different experiments. Scale bar: 20 μm.

After culturing the electroporated cells under neuronal differentiating conditions without any additional growth factors, both the GFP and Sox11 overexpressing cells started to express the immature neuronal marker DCX. In Sox11 overexpressing AHPs ~63% of Sox11 positive cells were DCX double positive whereas just ~24% of the GFP positive cells were DCX double positive (Figure 4.7B). Therefore one can conclude that Sox11 overexpression induces DCX expression and promotes neuronal differention in AHPs.

To reveal the effect of Sox11 on AHPs that were kept under enhanced neuronal differentiating conditions including forskolin (Fsk) and fetal bovine serum (FBS) immunostaining and counting procedures were performed as previously described.

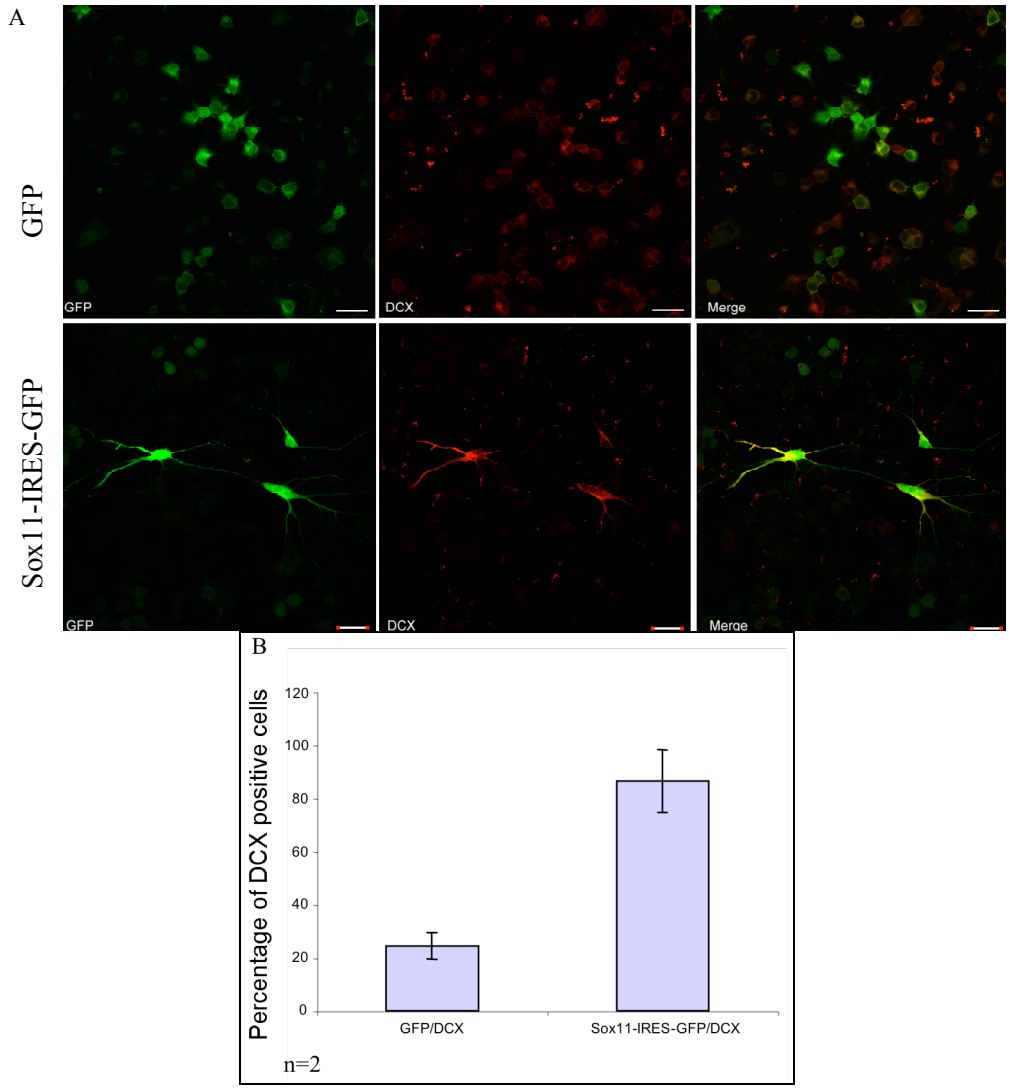


Figure 4.7: Sox11 overexpression induces DCX expression of AHPs kept under enhanced differentiating conditions. Electroporated AHPs were kept one day under proliferative conditions +FGF2 followed by three more days under neuronal differentiating conditions –FGF2, +Fsk and +serum. Four days after electroporation, cells were fixed and immunostained with GFP and DCX specific antibody (A). A quantification of GFP/DCX and Sox11-IRES-GFP/DCX double positive cells is shown in (B). Error bars indicate standard deviation of two different experiments. Scale bar: 20 µm.

In Sox11 overexpressing AHPs, ~86% of Sox11 positive cells were DCX double positive whereas just ~25% of the GFP positive cells were DCX double positive. Compared to the –FGF2 condition, there was an even higher induction of DCX

expression by Sox11 under enhanced differentiating condition. Therefore the addition of forskolin and serum has an additive effect on DCX expression in Sox11 overexpressing AHPs.

4.3.3 Viral overexpression of Sox11 induces DCX expression

To confirm the Sox11 overexpression results by a second approach, Sox11-IRES-GFP or GFP expression retroviruses were prepared and transduced into the AHPs. Retrovirus packaging of expression vectors was performed in HEK 293T cells. The titers of the Sox11-IRES-GFP or GFP retroviruses were both ~$1.8x10^5$ virus particle/ml. AHPs were plated with a density of $5x10^4$ cells/well in 24 well format including glass coverslips. After transduction cells were then kept overnight under +FGF2 proliferating conditions and then for three more days under –FGF2 differentiation conditions. After the cells were fixed with 4% paraformaldehyde, they were immunostained with GFP and DCX specific antibodies. The percentage of GFP/DCX and Sox11-IRES-GFP/DCX were calculated.

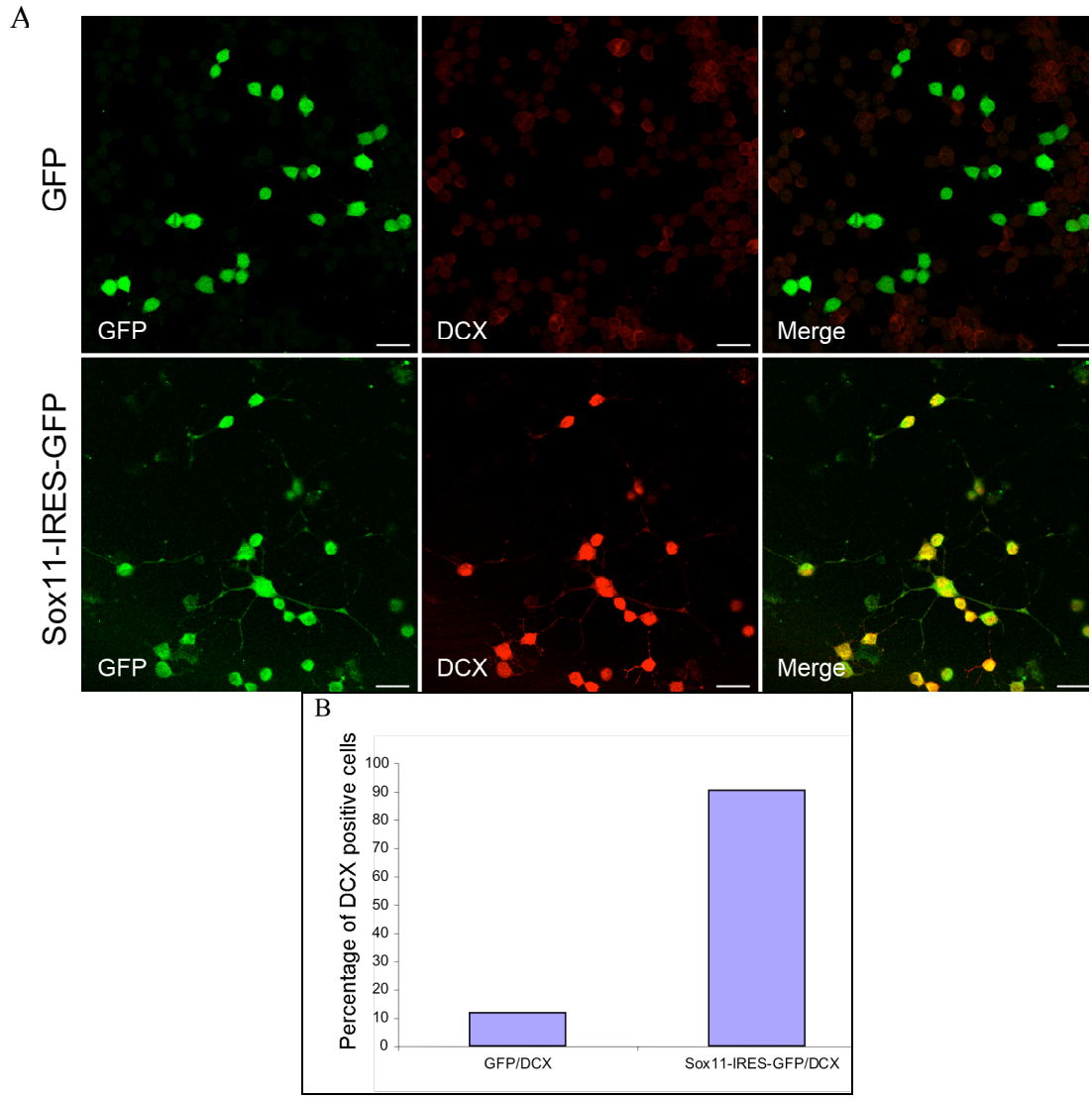


Figure 4.8: Viral overexpression of Sox11 induces DCX expression of AHPs kept under -FGF2 conditions. AHPs were transduced with Sox11-IRES-GFP and kept overnight under proliferative conditions (+FGF2), followed by 3 more days under neuronal differentiating conditions (–FGF2). 4 days after transfection, cells were fixed and immunostained for GFP and DCX (A). A quantification of GFP/DCX and Sox11-IRES-GFP/DCX double positive cells is shown in (B). Scale bars: 20 μm.

Because of using retroviruses, Sox11 and GFP constructs were transduced only into the dividing precursors. After keeping the cells under neuronal differentiation condition, both GFP and Sox11 transduced cells started to express the immature neuronal marker DCX. In Sox11 overexpressing AHPs, approximately 90% of Sox11 positive cells were also DCX double positive whereas only ~11% of the GFP positive cells were DCX double positive (Figure 4.8B). This different experimental approach also confirmed the previously shown results that Sox11 overexpression induces DCX expression and promotes neuronal differentiation.

4.3.4 Analysis of Sox11 effect on Tuj1 partial promoter sequences

There are three potential Sox11 binding sites on the Tuj1 promoter sequence identified so far. Bergsland *et al.* (2006) showed that Sox11 induces mouse partial Tuj1 promoter (from -566 to +54) ~9 fold in a LacZ reporter assay. To confirm this result, three rat Tuj1 partial promoter sequences (490F: from -490 to +1, 131F: from -131 to +1, 131R: from +1 to -131) in the pCAT vector (old version) were used (Dennis *et al.*, 2006). Since CAT analysis requires radioactive labeling, a luciferase assay was preferred for promoter analysis. For this part, promoter sequences were sub-cloned into pGL3 basic vector. Since there were no suitable restriction sites to ligate fragments directly into the pGL3 vector in 5'-3' orientation, the pKSPS vector was used to shuttle the fragments. The pCAT vectors containing the Tuj1 partial promoter sequences and the pKSPS empty vector were digested with HindIII/XbaI enzymes. Digested pKSPS vector and partial promoter fragments were extracted and purified and then ligation reaction was performed. Afterwards, the ligation mix was transformed to Top10 competent bacteria and small amounts of plasmid DNA of selected clones were prepared. DNA was digested with HindIII/XbaI enzymes to select the clones that contain the insert of interest. Inserted fragments have a length of 490 bp (490F) or 131 bp (both 131F and 131R) and the pKSPS shuttle vector has a size of 3 kb. Digested samples were analyzed on a 1% agarose gel. Only one of the positive clones that contain the right insert in the shuttle vector was selected for each fragment (Figure 4.9).

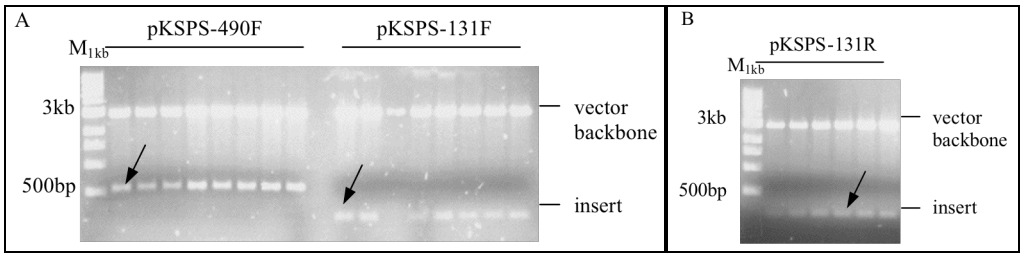


Figure 4.9: Test digestions of the partial Tuj1 promoter sequences ligated into the shuttle vector. XhoI/HindIII digestion of the (A) 490F, 131F and (B) 131R fragments ligated into pKSPS. Positive clones (arrows) that show a band at the right size were selected for further cloning. M_{1kb}: 1kb DNA marker (NEB).

To subclone these fragments into the pGL3 basic vector, 490F-, 131F- and 131R-pKSPS and empty pGL3 were digested with SmaI/XhoI enzyme. Gel extracted and

purified 490F, 131F and 131R inserts were then ligated into the digested pGL3 vector. Afterwards the ligation reaction was again transformed into Top10 competent cells and small amounts of plasmid DNA of selected clones were prepared. Purified DNA was digested with SmaI/XhoI enzymes to select the clones that contain the insert of interest. One of the positive clones that contain the right insert was selected for each of the three fragments (Figure 4.10).

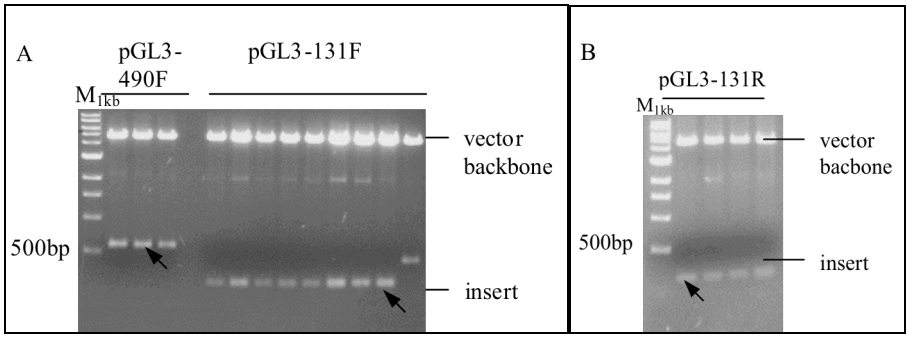


Figure 4.10: Test digestion of the partial Tuj1 promoter sequences ligated into the luciferase vector pGL3. SmaI/XhoI digestion of the (A) 490F, 131F and (B) 131R fragments ligated into pGL3. Positive clones (arrows) that show a band at the right size were selected for luciferase experiment. M_{1kb}: 1kb DNA marker (NEB).

These positive clones of the 490F, 131F and 131R fragments in the pGL3 vector were used in luciferase assays to analyze the effect of Sox11 effect on the Tuj1 promoter.

The 490F construct has 3 potential Sox11 binding sites whereas the 131F has only 1 Sox11 binding site (Bergsland *et al.*, 2006). These constructs, which are driving a firefly luciferase gene in the pGL3 basic vector, were together with Sox11-CAG-IRES-GFP transiently transfected into HEK 293T cells. The 131R construct that is the reverse oriented form of the 131F construct was used as a negative control. A TK-*Renilla*-luciferase was included to each transfection to normalize the firefly luciferase activity. The data were analyzed using CAG-IRES-GFP as control together with each pGL3 vector included promoter sequences transfected HEK 293T cells. Normalized firefly luciferase values were again normalized depending on the negative control. As it is shown in the luciferase assay data in Figure 4.11, Sox11 conrtaversly to the previously published data, did not significantly interact with the partial promoter sequences.

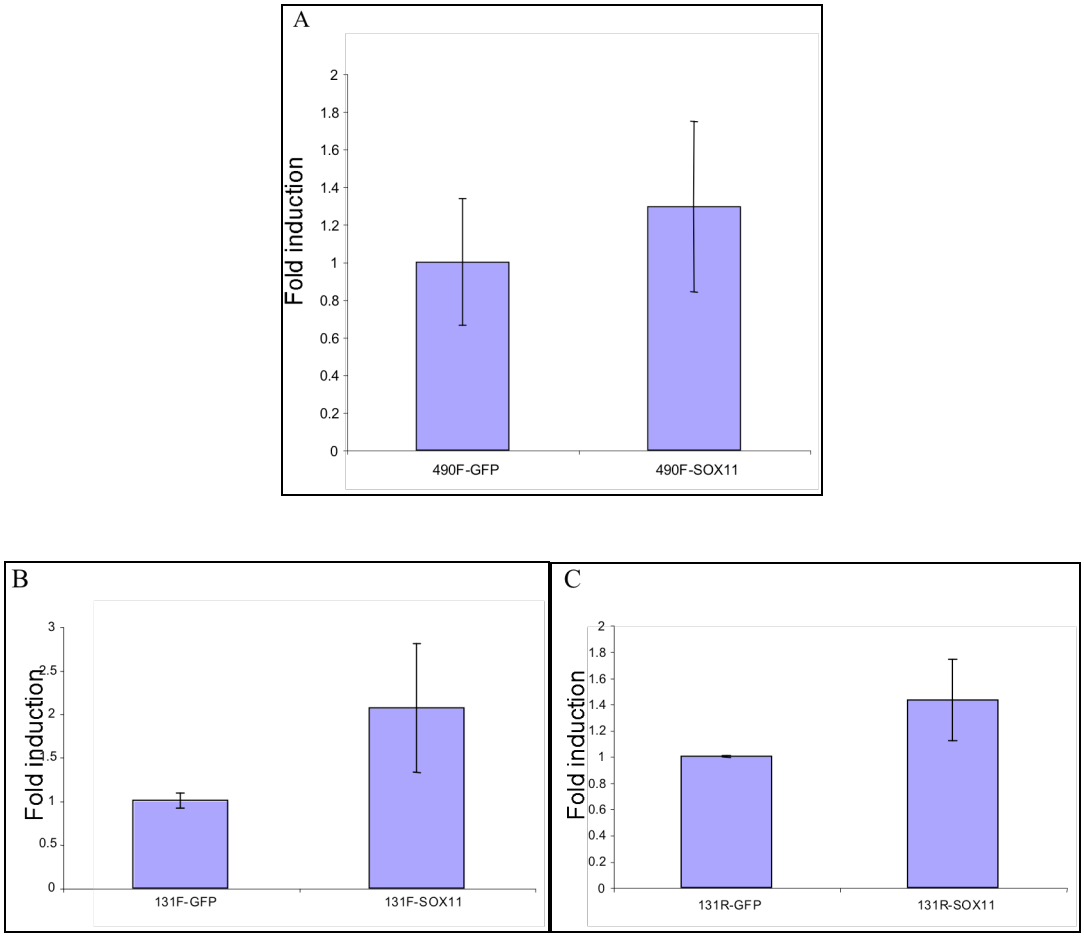


Figure 4.11: Sox11 effect on Tuj1 partial promoter sequences detected in a luciferase assay. Luciferase assay results of HEK 293T cells transfected with Sox11 and Tuj1 partial promoter sequences. Sox11 did not significantly induce the 490F fragment which includes 3 Sox11 binding sites (A). Sox11 induced 131F sequence 2 fold (B), 131R construct was used as an internal control of 131F (C).

4.4 Sox11 loss of function analysis

In order to analyze the role of Sox11 with loss of function studies, shRNAs against Sox11 were generated and functionally tested.

4.4.1 shRNAs were designed to knock down Sox11

Since interference RNA has the post-transcriptional gene silencing facility, shRNAs designed to knockdown Sox11. 4 different shRNAs with a length of 19 bases (N19) were designed to specifically knock down Sox11. shRNA primers have a loop sequence included in between sense and anti sense strand of the N19 sequence and a terminator sequence in the 5' end. Reverse primers were designed with a 5' sticky

end for XhoI. Reverse and forward primers were annealed together and cloned into the pLentiLox 3.7. pLentiLox 3.7 vector was digested with HpaI and XhoI and then ligated with the shRNA primers which contain a blunt end and an XhoI sticky end. After the ligation reaction the reaction mix transformed into Top10 cells and small amounts of DNA was prepared of the clones. If the shRNA primers are correctly ligated into the pLentiLox 3.7 vector the fragments shift on an agarose gel about 50 bp compared to the empty vector after digestion with XbaI and NotI. Therefore, the inserted fragments that contain Sox11 shRNA were running at ~500 bp whereas the insert that does not contain shRNA were at ~450 bp levels of the marker. The vector backbone alone has a size of 7.2 kb. Six clones were tested for each primer pair. Digested DNA were analyzed on 2% agarose gel. Two positive clones for each shRNAs were sequenced and used for further experiment (Figure 4.12).

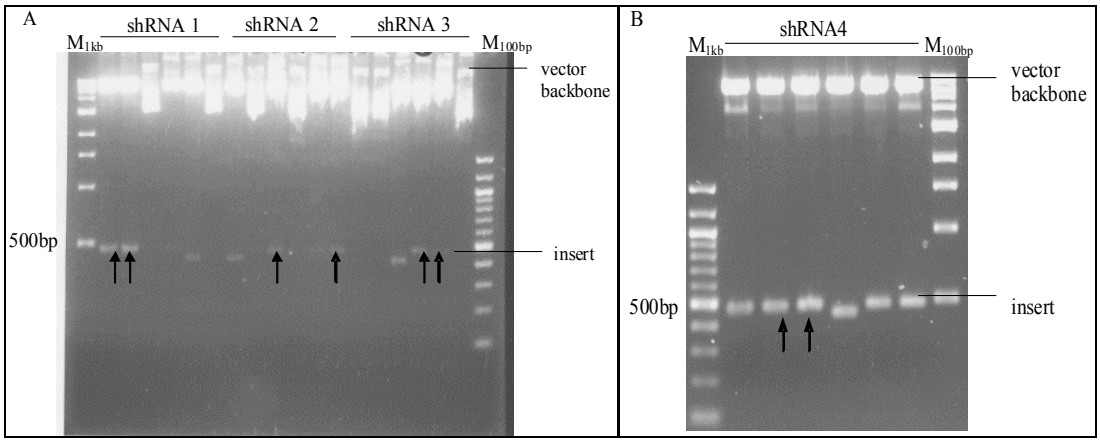


Figure 4.12: Test for positive clones containing Sox11-shRNA in the pLentiLox 3.7 vector. Test digestion of Sox11-shRNA 1, 2 and 3 (A) and Sox11-shRNA 4 (B) in the pLentiLox 3.7 vector with XbaI/NotI enzymes. Positive clones have an expected size of 500 bp (arrows), negative clones a size of 450 bp. M_{1kb}: 1kb DNA marker (NEB), M_{100bp}: 100bp marker (NEB).

4.4.2 Functionality analysis of Sox11-shRNAs on Western blot

To test for the functionality of designed Sox11-shRNAs, the constructs were transfected into HEK 293T with a full-length construct Sox11-CAG. Confluent HEK 293T cell plates with a diameter of 10 cm were transfected with a total of 5 μg DNA with the Calcium Phosphate method. A negative control was included into these experiments to guarantee the specificity of the designed Sox11-shRNAs. For this purpose HEK 293T cells were transfected either with the empty pLentiLox 3.7 vector

or with the GFP vector in combination with Sox11-CAG. For transfection reaction, equal amounts of Sox11-shRNAs and Sox11-CAG were transfected (2.5 μg). 48 hours after transfection, cells were lysed and total protein was isolated to be analyzed by Western blot. Sox11 antibody and beta-actin antibody that recognizes total amount of protein were used.

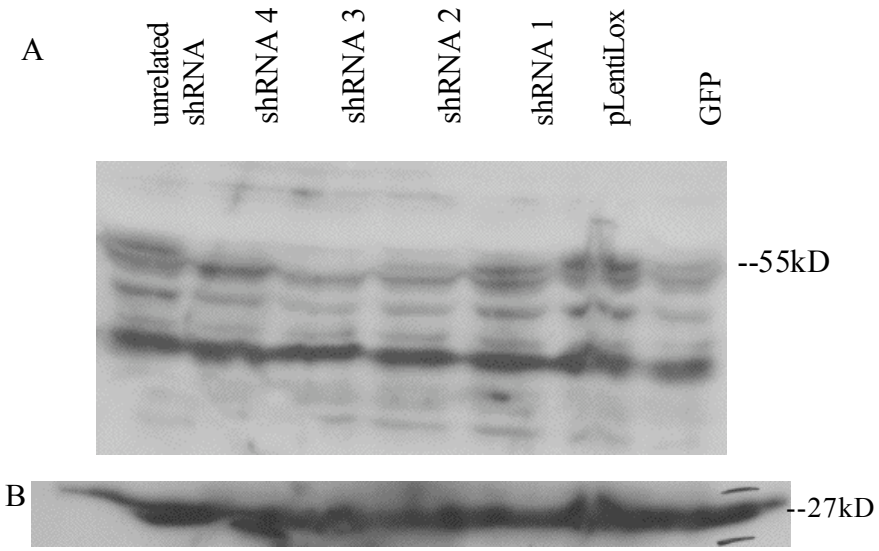


Figure 4.13: Knock down of Sox1 *in vitro* detected by Western blot. HEK293T cells were transfected with Sox11-IRES-GFP-CAG and four different Sox11-shRNAs. Transfection of either GFP alone, Sox11-Flag-CAG in combination with either empty pLentiLox3.7-GFP or unrelated shRNA served as negative controls. (A) Sox11 detection using a specific Sox11 antibody and (C) transfection efficiency control with total GFP detection with GFP antibody.

As shown in Figure 4.13 Sox11 specific products are reduced at the size of 55 kD in Sox11-shRNA transfected cell lysates. Again, the Sox11-shRNA2 and 3 seem to be the most efficient ones. The Sox11 specific product in control conditions like after transfection of an unrelated shRNA or an empty pLentiLox 3.7-GFP vector is not reduced compared to Sox11-shRNA transfections. As a loading control a GFP antibody staining was performed. This verifies that expression of transfected constructs is equal. Therefore this experiment confirms that Sox11-shRNA 2 and 3 are functional to knockdown Sox11 *in vitro*.

To verify the results shown with Sox11 antibody, the constructs were transfected into HEK 293T with a full-length construct Sox11-Flag-CAG. A negative control was included into these experiments to guarantee the specificity of the designed Sox11-shRNAs. For this purpose HEK 293T cells were either transfected with the empty

pLentiLox 3.7 vector in combination with Sox11-Flag-CAG. For transfection reaction, equal amounts of Sox11-shRNAs and Sox11-Flag-CAG were transfected (2.5 μg). 48 hours after transfection, cells were lysed and total protein was isolated to be analyzed by Western blot. Flag antibody that recognizes the flag-tag of Sox11-Flag-CAG and beta-actin antibody that recognizes total amount of protein were used.

As it is shown in Figure 4.14, Sox11-Flag protein was detected around 55 kD. The intensity of the Sox11-Flag bands were reduced in Sox11-shRNA transfected cell lysates especially in the case of Sox11-shRNA 2 and 3. This experiment shows that shRNA 2 and 3 are functional to knockdown Sox11-Flag in HEK 293T cells.

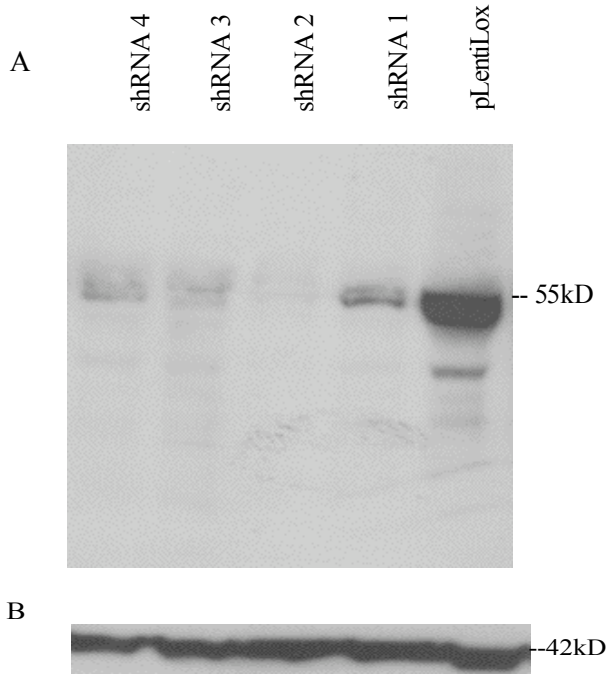


Figure 4.14: Knock-down of Sox11 in HEK cells detected by Western blot. HEK 293T cells were transfected with Sox11-Flag-CAG and four different Sox11-shRNAs. Transfection of Sox11-Flag-CAG in combination with empty pLentiLox 3.7 served as negative control. (A) Sox11 detection with a Flag antibody and (B) total amount of protein detection with beta actin antibody.

4.4.3 Functional analysis of Sox11-shRNAs with the psiCHECK vector system

To test the efficiency of the shRNAs for knockdown of Sox11 in a simple experimental set-up, the psiCHECK^{TM} from Promega (Germany) was chosen. This system relies on the measurement of a *Renilla*-luciferase, which is fused to the reporter-gen of interest. Therefore, the target sequence of Sox11 was subcloned into

the psiCHECK^{TM}2-expression vector. The psiCHECK^{TM}2 vector and the full length Sox11 coding sequence were digested with XhoI and NotI. Then the digested fragments were loaded on an agarose gel, purified and ligated. The ligation mix was transformed into Top 10 competent cells. Putative positive clones were again digested with XhoI/NotI to select for positive clones. The psiCHECK^{TM}2 vector has a size of 6.3 kb and Sox11 1.2 kb. Two of the selected clones contained the full length Sox11 sequence (Figure 4.15).

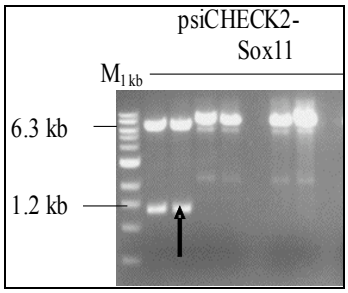


Figure 4.15: Test digest of the psiCHECK^{TM}2-Sox11. The putative positive clones were digested with XhoI and NotI. One of the positive clones (arrow) was selected to use in a further luciferase assay. M_{1kb}: 1kb DNA marker (NEB).

HEK 293T were transfected with 0.25 µg of the targeting sequence (psiCHECK^{TM}2-Sox11) and 0.25 µg of the respective shRNA (Sox11-sh-RNA-pLentiLox 3.7 or empty pLentiLox 3.7) to analyze the functionality of the Sox11-shRNAs in a luciferase assay. If shRNA led to the cleavage of the fused *Renilla*-targeting sequence, the signal of the *Renilla*-luciferase was decreased, since the cleavage of the chimeric mRNA resulted in a fast degradation of the whole mRNA molecule. The signal of the *Renilla*-luciferase was normalized against the firefly-luciferase to allow a qualitative analysis of the shRNA-efficiency after measuring the luciferase activity.

4 Sox11 specific shRNAs and an unrelated shRNA as control were tested for knockdown of Sox11. Forty-eight hours post-transfection *Renilla* and firefly luciferase activities were measured using the Dual-Luciferase Reporter 1000 Assay (Promega, Germany). The normalized Renilla-luciferase activity of psiCHECK^{TM}2-Sox11 co-transfected with the empty pLentiLox 3.7 was set to 1 %. In relation to this Sox11-shRNA 1 reduced the expression of the target gene Sox11 up to 20%, Sox11-shRNA 2 up to 40%, Sox11-shRNA 3 up to 62% and Sox11-shRNA 4 up to 25%

(Figure 4.16). Therefore, especially Sox11-shRNA 2 and 3 led to an efficient degradation of Sox11 *in* vitro, whereas Sox11-shRNA 1 and 4 are not sufficient to reduce the expression of Sox11 as shown in comparison with the unrelated shRNA.

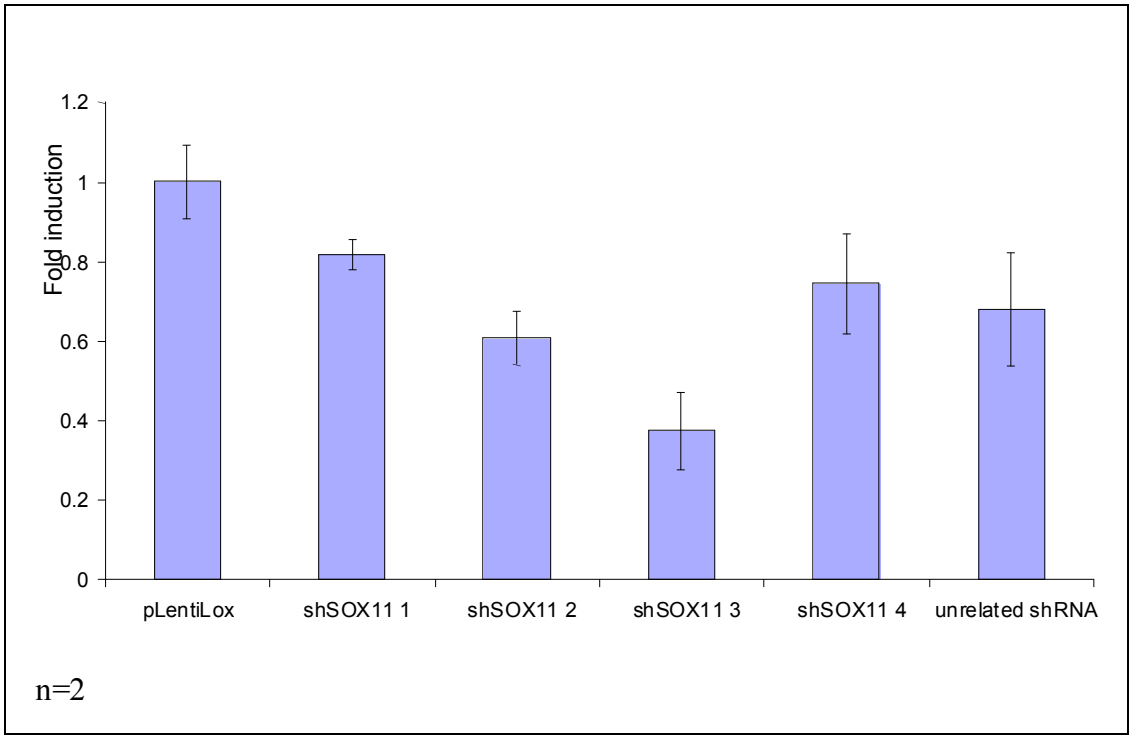


Figure 4.16: Selection of the target sequence to knock-down Sox11 with the psiCHECK^{TM}2 vector. Especially Sox11-shRNA 2 and 3 led to an efficient degradation of Sox11. Sox11-shRNA 1 reduced the expression of the target gene Sox11 up to 20%, Sox11-shRNA 2 up to 40%, Sox11-shRNA 3 up to 62% and Sox11-shRNA 4 up to 25%.

5. DISCUSSION & CONCLUSION

Neurogenesis is a continuous process in most of the mammalian brains throughout the life that is demonstrated in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ). This dynamic process is regulated by both extrinsic and intrinsic factors. Although neurotransmitters, growth factors and some signaling pathways regulate neurogenesis extrinsically, several transcription factors play an important role on its regulation intrinsically.

Aim of this study was to analyze the function of SRY box related high mobility group box (Sox) transcription factor Sox11 in neurogenic lineages in order to get a better understanding of adult neurogenesis regulation. Sox group transcription factors have regulatory functions during vertebrate CNS development. For instance the group B Sox proteins Sox1-3 were shown to keep neural cells undifferentiated by counteracting the activity of proneural proteins (Bylund *et al.*, 2003; Graham *et al.*, 2003). Whereas the SoxC group proteins Sox11 and Sox4 might act as a regulator of later steps neurogenesis in embryo because both are expressed in neural progenitor cells that have already been fate committed to neuronal differentiation (Uwanogho *et al.*, 1995; Bergsland *et al.*, 2006). It is also known that high expression of Sox2 in undifferentiated cells of embryonic neural epithelium is decreased when the cells leave the epithelium and get differentiated into neurons, whereas Sox11 expression in this cell population increases when the cell get differentiated into neurons (Uwanogho *et al.*, 1995). These data of the restricted expression pattern of Sox2 and Sox11 point out the possibility that Sox11 might control the switch from proliferation to neuronal differentiation during embryonic development. However up to now it was unknown if Sox11 also affects adult neurogenesis.

Sox11 is expressed by newly born immature neurons in the RMS of the adult brain

Sox11 is expressed in the olfactory bulb (OB) and hippocampus as shown by RT-PCR and by immunohistochemical staining. It is known that there are type A immature migrating precursors in the RMS that express doublecortin (DCX) and ß-III-tubulin (Tuj1). When the Sox11 staining was performed in combination with DCX and Tuj1, most of the Sox11 expressing cells were Tuj1 and/or DCX positive.

DCX is a microtubule-associated protein specifically and transiently expressed in all migrating proliferating neuronal progenitors and in post-mitotic neuronal precursors (Brown *et al.*, 2003; Rao and Shetty, 2004; Coillard-Despres, 2001). On the other hand, Tuj1 is a neuron specific microtubule monomer transiently expressed in immature neuroblasts that acts on microtubule dynamicity (Roskams *et al.*, 1998). Therefore we can conclude that in the RMS Sox11 is expressed in migrating neuronal precursors called type A cells.

Sox11 is expressed by newly born immature neurons in the hippocampus of the adult brain

RT PCR and immunohistochemical staining also revealed Sox11 expression in the hippocampal dentate gyrus. It was observed in the hippocampus that all of the Sox11 positive cells were not positive for Sox2 or GFAP. In other words, there might be an on/off mechanism between Sox2 and Sox11. Sox2 expression might maintain stemness such as type 1 and 2a cells do, whereas Sox11 expressing cells might be related in fate specification such as type 2b and 3 are. So Sox11 expression might be important for lineage determination.

It was shown that Sox11 immunoreactive cells were immature neurons that were also labeled with Tuj1, DCX and calretinin which are immature neuronal markers. In hippocampus, type 2b transiently amplifying progenitors express DCX and type 3 lineage determined post-mitotic migrating precursors express both DCX and Tuj1. DCX expression is related to the entry of neuronal differentiation and migration (Francis *et al.*, 1999). The expression pattern of Tuj1 is not known but its association with immature neurons is identified (Uittenbogaard and Chiaramello, 2002). Additionally, calretinin positive cells are post-mitotic and immature granule cells. Sox11 expression in both mitotic and post-mitotic cells represents that Sox11 does not involve in cell cycle exit (Bergsland *et al.*, 2006).

Lastly, Sox11 positive cells did not express calbindin that is a mature neuronal marker. We can say that Sox11 is not expressed in mature neurons.

In conclusion, Sox11 expression pattern in neurogenic lineages shows that Sox11 expression promotes neuronal differentiation. It might have a role in transition from multipotent stem cells to fate specified neuroblasts.

Sox11 highly activates the DCX promoter

The overlapped expression pattern of Sox11 and DCX leads to the question if Sox11 acts upstream of DCX. It was observed by HEK 293T cells transient transfection that

the DCX promoter was highly activated by just 8 ng Sox11/ $3x10^6$ cells. *In silico* analyses revealed that the DCX promoter contain Sox11 specific binding sequences. Although it is a possibility that Sox11 might bind to these sequences to activate DCX promoter, binding assays such as chromatin immunoprecipitaion should be done to clarify Sox11 binding sites on DCX promoter. In a dose dependent experiment it was shown that even very small amounts of Sox11 are sufficient to activate the DCX promoter. Hence, Sox11 might be an upstream element of DCX. Additionally these results are supported by the observation that the staining of Sox11 is significantly weaker compared to DCX.

Tuj1 promoter analysis with Sox11

It was previously shown that Sox11 activates the upstream segment of the mouse Tuj1 promoter (Bergsland et al., 2007). To confirm this experiment Tuj1 promoter 5' flanking regions were analyzed if Sox11 effects on them. Both 490 bp and 131 bp upstream regions of the rat Tuj1 gene were shown to be sufficient to drive the expression of a reporter gene in embryonic stem cells during neuronal differentiation were used (Dennis *et al.*, 2002). In contrast to the results shown by Bergsland *et al.*, there was less activation of Tuj1 promoter segments by Sox11. Although three Sox11 binding sequences were identified on 490 bp upstream and one binding sequence was identified on 131 bp upstream of the Tuj1 gene, Sox11 did not highly activate the promoter segments in HEK 293 cells. This experiment was done just one time. To get a significant result it should be repeated at least 3 times.

Overexpression of Sox11 induces DCX expression so promotes neuronal differentiation in AHPs

In this study, Sox11 overexpression led to an induction of DCX expression in AHPs. Even AHPs overexpressing Sox11 that were kept under neuronal differentiating conditions without addition of any growth factor or serum exhibited an increased expression of DCX. This effect was even amplified when these cells were kept under differentiating conditions including forskoline and serum (FBS). Under these conditions Sox11 overexpression led an even stronger induction of DCX expression. These results were confirmed in two independent experiments either by electroporation or viral transduction of Sox11. Therefore it can be suggested that once the differentiation is started by external factors added to the media, Sox11 has an even higher induction capacity on DCX expression.

These experiments demonstrate that Sox11 might be a regulator of neuronal differentiation. It promotes neuronal differentiation by inducing DCX expression and

maybe additional factors that are not identified yet. It is known that DCX is involved in migration of immature neurons, so Sox11 might also be involved in migration step of neurogenesis together with DCX. For further step, Sox11 overexpression would be done *in vivo* with retrovirus injection to adult mouse hippocampus and then DCX antibody staining would be performed to see any effect on them. Furthermore after the viral injection, morphological changes of injected cells can also be analyzed. Because Sox11 is expressed in calretinin stage that is the period of dendrite and axon formation and functional maturation, it would be interesting to observe the injected cell electrophyisologically to test its functional integration. If Sox11 will be overexpressed *in vivo*, there might be more mature neurons developed by the activation of neuronal genes such as DCX in the DG. Furthermore Furthermore less immature neurons might be seen in the SGZ. Since the stem cell population would not be changed, more migrated and mature cells but less immature cells would be seen.

In this study, Sox11 was overexpressed with the electroporation of CAG-Sox11-IRES-GFP vector in AHPs. Sox11 expressing cells should have been detected by Sox11 specific antibody staining in the electroporated AHPs. Since Sox11 antibody staining was not specific *in vitro,* Sox11 expression was detected by GFP signal instead. The existence of GFP signal is not enough to prove that Sox11 is expressed in the cell. As the results of DCX promoter analysis would indirectly confirm the overexpression results, Sox11 expression was analyzed through DCX immunostaining based on Sox11 activating DCX promoter. For further experiments, specific Sox11 antibody staining should be performed to reveal the Sox11 expression more accurately.

Two out of four generated shRNAs against Sox11 are functional

Because the Sox11 gene have diverse role on embryonic development of different organs, Sox11 knockout mice dies at birth because of several organ defects (Sock *et al.*, 2004). There is no chance to use knockout mice for adult analyses. Thus, to generate a tool for loss of function analysis of Sox11, four different shRNAs that contains different target sequences against Sox11 were produced to knockdown specifically Sox11. It was demonstrated by Western blot and psiCHECK system that shRNAs 2 and 3 were more efficiently silencing Sox11. Other shRNAs might be instable in the cell or they might have secondary structures that prevent access to the target mRNA. Further step with produced functional shRNAs will be to knockdown Sox11 *in vivo* by using shRNAs and to test if they are specific for Sox11. Because HMG domain of another SoxC group protein Sox4 has a great homology with Sox11

(92% in aminoacid homology) (Wright *et al.*, 1993), the specificity of shRNA knockdown studies should be checked. After the Sox11 knock-down, its effects on neuronal differentiation would be analyzed. Sox4 and Sox11 have redundant functions (Lefebvre *et al.*, 2007) therefore it is possible that one might compensate deficiency of the other one. If Sox11 would be knocked down, transition from stem like cells into neuronal lineage determined immature neurons would be affected. There might be less immature neurons that are DCX or Tuj1 positive.

Briefly, this study demonstrated that Sox11 expression promotes neuronal differentiation in AHPs via inducing neuronal gene expression like DCX. Additionally, Sox11 might be an upstream regulator of DCX.

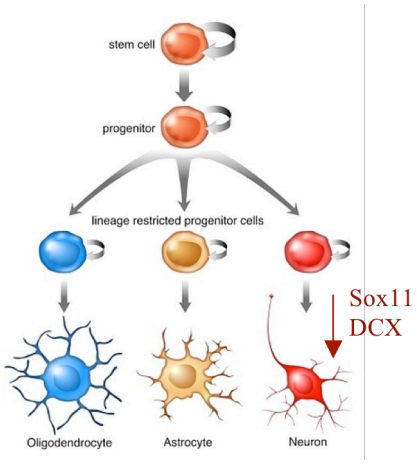


Figure 5.1: Schema of Sox11 effect on neuronal differentiation

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