

Oxygen-Glucose Deprivation Modelling and Overnight Incubation of
Acute Hippocampal Slices *in Vitro*

Master Thesis

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Declaration

This thesis was written at the Institute of Physiology and Pathophysiology at the Ruprecht-Karls-University of Heidelberg in the period from 10.11.2020 to 05.08.2021 under the supervision of Prof. Dr. med. Andreas Draguhn.

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I herewith declare that I wrote this Master Thesis independently, under supervision, and that I used no other sources and aids than those indicated throughout the thesis.

03.08.2021

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ABSTRACT

During hypoxia-ischemia, as occurring in ischemic stroke, neurons are deprived of oxygen and glucose, which within minutes leads to neuronal network dysfunction. Hippocampal pyramidal cells (PYR) and fast-spiking interneurons (FSI) are particularly vulnerable to oxygen-glucose deprivation (OGD). Previous studies on tissue from young adult mice have shown that the dysfunction of FSI may contribute to impaired network recovery following OGD. However, currently no data exists on aged mice, although this group is of particular interest as stroke primarily affects the elderly. The aims of this thesis are 1) to close this knowledge gap by studying the recovery of neuronal function from acute hypoxia-ischemia on cellular and network levels in acute brain slices from aged mice, 2) to compare the effects of mild vs. severe OGD on axonal morphology and 3) to extend the experimental time window in acute brain slices.

OGD was modelled in acute slices from 75- to 104-week-old mice by switching the perfusion and gas to an oxygen- and glucose-free medium, until a spreading depression (SD) was induced. SD was detected extracellularly by a large negative potential shift and a transient cessation of neuronal firing. Tetrode recordings were performed in the hippocampal CA1 and CA3 regions during OGD, until 60 min and after four hours of recovery to measure single-cell and neuronal network activity.

The hippocampus in our preparation spontaneously entrains its innate form of network activity, the sharp wave-ripple (SPW-R) oscillations, which are crucial for spatial and episodic memory formation. After one hour, and even more so after four hours of recovery SPW-R frequency was reduced in both CA1 and CA3 regions. At one hour of recovery FSI showed a reduced firing rate, while PYR spiking was not significantly altered. After four hours, FSI firing remained reduced compared to control in both regions, while PYR firing increased. Overall, unit firing of the CA1 region was less coupled to SPW-R compared to control.

As changes in the excitatory-inhibitory balance have been shown to be accompanied by changes in axon initial segment (AIS) morphology, slices were stained for β 4-spectrin, a marker of AIS, after experiments. However, no changes in AIS length nor numbers were detected after four-hour recovery from mild OGD. In contrast, a more severe OGD for 20 min severely diminished AIS numbers and length in a separate set of experiments.

In a next step, irradiation of artificial cerebrospinal fluid (ACSF) with ultraviolet (UV) light and addition of the antibiotics penicillin and streptomycin was used to extend the viability of

acute slices. After 21 hours experimental slices still had SPW-R activity in their CA3 region but not in their CA1 region and the control slices, which were perfused with normal ACSF without the added UV light nor antibiotics, showed no SPW-R activity in both regions. No difference in AIS length nor numbers were detected between experiment and control slices for the CA1 region.

Together, these results in tissue from aged mice show similar effects of OGD on FSI and SPW-R activity as shown in young mice in a previous study from our group and support the hypothesis of FSI dysfunction following mild hypoxia-ischemia. Furthermore, they suggest an increasing inhibitory deficit over time. While AIS morphology is unaltered after mild OGD, we show that AIS break down after severe OGD, which may be an important pathomechanism of ischemic neuronal damage. Furthermore, we prove that it is feasible to maintain acute slices expressing spontaneous SPW-R activity after 21 hours of incubation, which significantly widens the time window for experiments and enables the study of processes like delayed apoptosis and long-lasting changes in network activity.

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List of abbreviations

ACSF	Artificial cerebrospinal fluid
ACSF + PS	ACSF containing penicillin and streptomycin
AIS	Axon initial segment
a.u.	Arbitrary unit
Base.	Baseline
CA	Cornu Ammonis
Ca	Calcium
CO ₂	Carbon dioxide
Control 4h	Control slices at four hours post SD
DAPI	4', 6-diamidino-2-phenylindole
DG	Dentate gyrus
Fall-Norm	Normalized rate of fall
FSI	Fast-spiking interneuron units
INT	Interneuron
K	Potassium
L	Liter
LFP	Local field potential
Log of ISI	Logarithm of the inter spike interval
MIX	Mixed units
ml	Milliliter
mM	Millimolar
ms	Milliseconds
MUA	Multi-unit activity

mV	Millivolt
N ₂	Nitrogen
Na	Sodium
NeuN	Hexaribonucleotide Binding Protein-3
n.s.	Not significant
O ₂	Oxygen
OGD	Oxygen glucose deprivation
OGD 1h	OGD slices at one hour post SD
OGD 4h	OGD slices at four hours post SD
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
PV +	Parvalbumin positive
PV-tdTomato	PV + INT expressing tdTomato fluorescent protein
PYR	Pyramidal cell units
Rise-Norm	Normalized rate of rise
SD	Spreading depression
SPW-R	Sharp wave-ripple complex
tRightPeak	Trough-to-right-peak latency
UV	Ultraviolet
UVPS	Ultraviolet-penicillin-streptomycin set up
μm	Micrometer
Width75	75 % amplitude width
20x	20 times magnification
60x	60 times magnification

INTRODUCTION

1.1. Hypoxia-ischemia and spreading depression

Ischemic stroke, defined as a reduction of blood flow to brain tissue, resulting in decreased oxygen and nutrient supply to nerve cells, is one of the major causes of death (Vos et al. 2020). One of the characteristic events following the deprivation of oxygen and glucose is spreading depression (SD) (Somjen, 2001). It was first described in 1944 by Aristides Leão, who was studying the cortical electrogram (EcoG) following experimental epilepsy in anesthetized rabbits, when he discovered a silencing of electrical activity (thus a depression of neuronal activity) following the expected seizure. (Leão, 1944). This depression of neuronal activity spread throughout the cortex, showing itself in successive surface electrodes placed further from the origin of the depression, thus it was named spreading depression (SD) (Leão, 1944). Leão later observed this event in cortical tissue following hypoxia which occurred alongside a voltage shift of the extracellular field potential of approximately -15 mV, which is significantly larger than any voltage shift caused by regular brain waves (Leão, 1947).

The cause of this depression of neuronal activity is that as neurons are deprived of oxygen and glucose, they become unable to fire as they are deprived of energy. The cause of the sudden and large voltage shift of the local field potential is that after a certain time in hypoxic-ischemic conditions, neurons become unable to maintain their resting membrane potential. Their membranes are depolarized on mass and this depolarization spreads across the tissue (Figure 1). (Somjen, 2001). This depolarization is accompanied by a large out flux of K^+ ions and a large influx of Na^+ and Ca^{2+} ions (Somjen, 2001, Mattson et al. 2000). The influx of Ca^{2+} ions is thought to induce apoptotic cell death through several mechanisms such as an increase in free radicals or mitochondrial damage (Mattson et al., 2000, Dirnagl et al., 1999). Thus, it is thought that SD is an additional insult on neurons already suffering from hypoxic-ischemic conditions and it is considered as a hallmark of neuronal damage caused by hypoxic-ischemic conditions (Dreier, 2011). In one experiment it was found that around 40 % of the CA1 neurons of rabbit hippocampus were dead 2 days after an induced 10 minute forebrain ischemia and around 80 % of these neurons were dead 3 days after the 10 minute induced forebrain ischemia (Mattson et al., 2000).

Hippocampus is known to be especially vulnerable to brain ischemia (Schmidt-Kastner and Freund, 1991). Furthermore, it is known that ischemic stroke patients often suffer from memory deficits (Pendlebury and Rothwell, 2009), which suggest some form of damage to the

hippocampus neuronal network which has a significant role in memory formation (Buzsáki, 2015). Also, it was observed that following SD, neurons recover their membrane potential and their ability to fire action potentials, if SD is followed by re-oxygenation of the brain tissue (Heftner et al., 2016; Revah et al., 2019). However, little is known about the short-term or mid-term effect of this mild and reversible oxygen glucose deprivation (OGD) on network oscillations and single-cell activity. Along with these, aging is also known to be the single most decisive risk factor of incidental ischemic stroke (Yousufuddin and Young, 2019). So, it is valuable to perform induced mild OGD on the hippocampus of acute brain slices obtained from old mice, and record the electrophysiological short-term and mid-term (four hour recovery) effects on single cell activity and network oscillations, which is one of the primary experiments of this thesis.

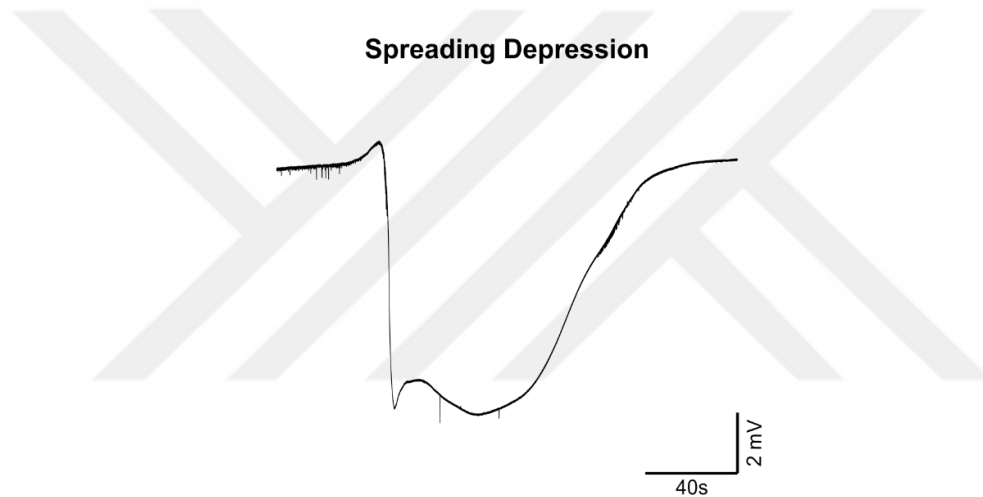


Figure 1: SD as recorded from the CA1 local field potential. Note the sudden downward shift and gradual recovery of the local field potential (LFP).

1.2. OGD models in acute brain slices and extending their viability

As written above, little is known about the short to midterm effects of mild OGD on neuronal network activity, which can also be said for the long-term effects of mild OGD on acute brain slices. As mentioned in the hypoxia section, 3 days after 10 minute global ischemia, up to 80 % of CA1 pyramidal cells of the rabbit hippocampus were dead, but even 1 day after the insult, up to 100 % of the CA1 pyramidal cells were still intact (Mattson et al., 2000). This finding shows the importance of extending the recovery period of acute brain slices from OGD by extending the viability of acute brain slices. For the mild and 20-minute OGD experiments performed on this thesis, the maximum recovery time was four hours following OGD and extending this time would allow tetrode recordings to be taken at 8, 16 or even 24 hours

following OGD which would definitely lead to more insight into the long-term effects of OGD on single cell and network activity.

After they were developed in the early 50's, and 60's (McIlwain et al., 1951, Yamamoto and McIlwain, 1966) *in vitro* brain slices became an invaluable tool in neuroscience experiments and they are still one of the most common methods employed in the field of neuroscience (Buskila et al., 2014). *In vitro* brain slices allow for various experiments but they have a significant disadvantage which is that acute brain slices are known to remain viable for 6-12 hours (Buskila et al., 2014). Some findings even suggest that many hippocampal and neocortical thin slices can only remain in a perfectly healthy condition with most cells functioning normally for up to four hours (Fukuda et al., 1995). This time limit prevents long-term experiments on acute brain slices and one of the main reasons for this time limit is the growth of harmful bacteria on the medium that is used to keep acute brain slices alive (such as ACSF) (Buskila et al., 2014). Since the acute brain slices are without an intact immune system, they are defenseless against bacteria (Buskila et al., 2014) and it was shown that bacteria reach their exponential growth phase 6-12 hours under regular artificial cerebrospinal fluid (ACSF) incubation conditions (Buskila et al., 2014, Terenzi et al., 1995, Knowles et al., 1990, Moncada and Higgs, 1995). One possible solution for this problem could be the addition of antibiotics to the ACSF, however, it is also known that many antibiotics increase the activity of neurons which may lead to epileptic episodes (Grøndahl and Langmoen, 1993).

In Buskila et al., (2014) researchers created a special submerged incubation chamber called the 'Braincubator' to keep the slices viable for up to 36 hours in a relatively cold (15-16 °C) ACSF medium which is circulated through a chamber containing ultraviolet (UV) light which sterilizes the ACSF (Figure 2). The reason to keep the slices in a relatively cold (15-16 °C) environment was to further curb bacterial growth (Buskila et al., 2014). The viability of the brain slices was assessed by staining the cells with the dead cell marker Propidium Iodide (PI) and measuring the ratio of dead cells, as well as measuring membrane potential properties of certain neurons. In both cases there was significant improvement compared to control slices (Buskila et al., 2014). However, there was no network oscillation recordings to assess whether network oscillations survive longer in these slices.

This method has potential to be adapted to the Haas-type interface chamber, however, some changes have to be made, most importantly the temperature of the surface of the interface chamber has to stay in between 32-36°C, as this range is known to be best suited for the generation of SPW-R oscillation in acute hippocampal slices (Maier et al., 2003, Maier et al.,

2009, Papatheodoropoulos and Kostopoulos, 2002, Behrens et al., 2005). Another possible change could be the addition of antibiotics to the ACSF since, even though UV lamp sterilizes the ACSF, the surface of the interface chamber can still sustain the growth of bacteria and the UV lamp can't be placed near the surface due to potential damage it would cause the slices. In this thesis, hippocampus slices of P60-70 mice were incubated for 21 hours in a modified Hass-type interface chamber set-up which will be explained more in detail in the methods section.

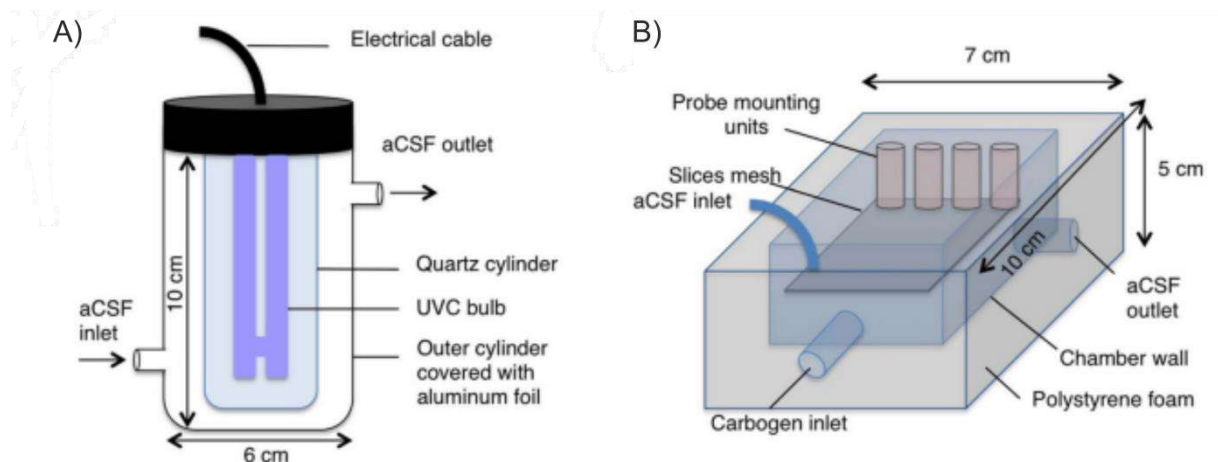


Figure 2: The incubation method used in Buskila et al. (2014). A) UV light chamber, with ACSF inlet and outlet, B) The main chamber ('Brincubator') used to keep slices submerged in ACSF at 15-16 °C. Figure adapted from (Buskila et al., 2014).

1.3. The role of hippocampal SPW-R in memory formation and putative mechanisms of their generation

Hippocampus as a brain structure has been implicated to take part in processes such as memory formation and consolidation (McGaugh, 2000, Scoville and Milner, 1957) but also is thought to have a crucial part in anxiety, fear and stress responses (Bartsch and Wulff, 2015) and planning (Buzsáki, 2015). It is also hypothesized that hippocampus works as a filter to distinguish information that is novel to the organism, as a novelty detector (Vinogradova, 2001). Hippocampus is a highly compartmentalized brain structure and has four main regions, the dentate gyrus (DG), the Cornu ammonis (CA) with the sub-regions CA1, CA2 and CA3, the subiculum and the entorhinal cortex (EC) (Amaral and Witter, 1989) (Figure 3). The CA and dentate gyrus regions of the hippocampus contain a large number of pyramidal cells and in the CA region, the cell bodies of the pyramidal cell are packed closely together in the pyramidal cell layer (stratum pyramidale) (Draguhn et al., 2000). 5 to 10 % of the neurons of the hippocampus network is thought to consist of interneurons that use the neurotransmitter g-

aminobutyric acid (GABA) to mainly inhibit the pyramidal cells of the CA and DG regions (Draguhn et al., 2000; Freund and Buzsáki, 1996).

There are two main information relaying pathways of the hippocampus, the tri-synaptic pathway sends signals to the DG region by the perforant pathway (pp) and the granule cells of the DG, relay this signal to the CA3 pyramidal cells through mossy fibers (Andersen et al., 2007). CA3 pyramidal cells connect to the CA1 pyramidal cells through the Schaffer collaterals (Andersen et al., 2007) (Figure 1). The monosynaptic pathways relays information from the EC, directly to the CA1 pyramidal cells, bypassing the DG and CA3 networks, and in both the tri-synaptic and mon-synaptic circuits, the final step is the relaying of information from the CA1 region to the subiculum and there on to the deeper regions of the EC (Andersen et al., 2007).

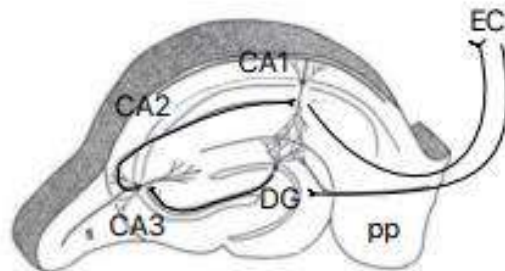


Figure 3: Hippocampal regions and the two circuits. Figure shows the tri-synaptic and the monosynaptic circuits of the hippocampus. DG= Dentate gyrus, pp = perforant pathway, EC= entorhinal cortex (EC), CA = Cornu ammonis. Figure adapted from (Anderson et al., 2007).

Sharp waves are large and slow network oscillations of the local field potential that are caused by the simultaneous activity of a large number of neurons of the hippocampus (Schlingloff et al., 2014). They are positive or biphasic waves of 0.05 to 0.5 mV amplitude, lasting around 30-80 milliseconds (Maier et al., 2003) (see Figure 11, A for an exemplary image of SPW-R) that are known to initiate in the CA3 region and propagate through to the CA1 region, and subsequently leave the hippocampus neuronal circuit through the subiculum and the entorhinal cortex (Schlingloff et al., 2014, Both et al., 2008). SPW-R can be generated in the CA1 region by stimulating the CA3 region but not vice-versa, showing that CA1 sharp waves are strongly coupled to and determined by the neuronal activity of the CA3 region (Both et al., 2008). Sharp waves are usually accompanied by a fast oscillation (around 150-250 Hz), and these are called ‘ripples’ (Schlingloff et al., 2014, Both et al., 2008, Draguhn et al. 2000).

Together the sharp wave oscillations superimposed with faster ripple oscillations are termed the sharp wave-ripple complex (SPW-R), and the firing of neurons in the hippocampus are strongly coupled to these SPW-R and this in turn facilitates temporal coding by these neurons (Schlingloff et al., 2014, Both et al., 2008). Recently, it was found that during sleep cycles, the CA1 place cells of rats' fire in the same temporal sequence as they did during a learning event, and this sequential firing occurs within SPW-R complexes in a time-compressed manner. (Lee and Wilson, 2002).

There is a distinct interplay of excitatory pyramidal cells and inhibitory interneurons in the formation and propagation of the SPW-R. (Schlingloff et al., 2014, Evangelista et al., 2020). It is thought that SPW-R occur initially in the CA3 region if there is a sufficient co-activity of CA3 pyramidal cells which then through a recurrent excitatory network, cause a build-up of activity which is the sharp wave oscillation (Schlingloff et al., 2014). The sporadic nature of this build-up of initial activity suggests that as the neuronal network grows larger and more active, the chance of sharp wave initiation increases, which can be one key reason why acute hippocampal slices thinner than 350 μm rarely show any SPW-R activity (Schlingloff et al., 2014).

After the initiation of a sharp wave, excitatory pyramidal cells propagate this oscillation through to CA1 while simultaneously activating interneurons, parvalbumin positive basket cells (PV+ interneurons) being of special importance (Schlingloff et al., 2014). These PV+ interneurons form reciprocally connected networks with each other and upon activation by pyramidal cells, start rapidly firing and inhibiting neighboring PV+ interneurons. This leads to a fast oscillation which is termed as fast inhibitory neuronal oscillation (FINO) and is thought to be the principal component of the ripple oscillation (Schlingloff et al., 2014, Evangelista et al., 2020) (Figure 4). These interneurons also inhibit pyramidal cells with the same frequency of the FINO, creating the strong coupling of pyramidal cell firing to SPW-R complexes (Schlingloff et al., 2014). Cutting the reciprocal connections between PV+ interneurons leads to a desynchronization between ripple oscillation and sharp waves (Schlingloff et al., 2014). It was also found that optogenetic activation of PV+ interneurons initiates SPW-R and that optogenetic silencing of these neurons lead to silencing of the SPW-R, showing the importance of these cells for SPW-R (Schlingloff et al., 2014).

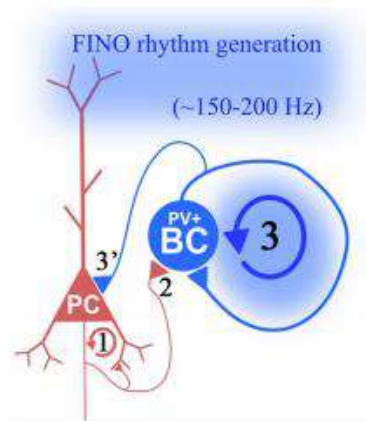


Figure 4: Representation of SPW-R generation and the FINO rhythm. 1=Recurrent excitation network of pyramidal cells (PC) cause a build-up of activity which initiates the sharp wave, 2= Activation of PV+ basket cells (PV + BC) by the pyramidal cells, 3=Reciprocal activation of the PV+ BC network which creates the FINO oscillation, 3'= PV+BC cells inhibit pyramidal cells in accordance with the FINO oscillation. Figure adapted from (Schlingloff et al., 2014).

1.4. Interneuron vulnerability to metabolic stress

As explained in the previous section, interneurons play a key role in the formation and maintenance of fast-oscillations of neuronal circuits such as ripples by firing with high frequency (150-200 Hz) within their reciprocal network (Schlingloff et al., 2014). Interneurons expend large amounts of energy to maintain this fast-spiking and also to maintain fast release of the neurotransmitter GABA which inhibits the surrounding pyramidal cells. Hence, interneurons were found to be enriched in mitochondria and cytochrome c oxidase, reflecting their increased demand of oxidative phosphorylation (Kann, 2016).

Interneurons also play a key role in the gamma oscillations (30-100 Hz) that occur during higher cognitive functions, such as attention, sensory perception or memory formation (Lisman, 1999; Axmacher et al., 2006; Mann and Paulsen, 2007; Uhlhaas and Singer, 2010). It was found that during gamma oscillations, the utilization of mitochondrial oxidative capacity was almost at the limit (Kann, 2012, Kasischke et al., 2004; Brennan et al., 2006; Hertz et al., 2007, Kann et al., 2011). This finding indicates that fast oscillations such as gamma and ripple oscillations are especially affected by metabolic stress and the high energy demand of interneurons might set a threshold on the metabolic stress the hippocampal neuronal network can endure before fast oscillations are disrupted (Kann, 2016).

Since interneurons have higher energy demands, it is expected that they would be more vulnerable to oxidative or metabolic stress than pyramidal cells and this was found to be the

case following OGD and re-oxygenation of mouse prefrontal cortex slices, where a lower percentage of PV + interneurons recovered than pyramidal cells following OGD (Povysheva et al., 2019). Inducing mild metabolic stress to the CA3 region of rat hippocampal slices by injecting rotenone, an inhibitor of mitochondrial function, attenuated gamma oscillation and decreased the inhibition of slow-spiking units (putative pyramidal cells) and lead to a significant increase in the frequency of firing of these slow-spiking units (Elzoheiry et al., 2020). In a recent experiment, mild OGD was performed on mice hippocampal slices, and it was found that at one hour post SD, fast-spiking interneuron firing was reduced more strongly than the pyramidal cell firing. (Grube et al., manuscript in print).

These various findings indicate the importance of analyzing the effect of mild OGD separately for fast-spiking interneuron and pyramidal cell firing in order to observe the extent of the selective vulnerability of interneurons. In this thesis, this is achieved by clustering the spikes of the electrophysiological recordings into different units which are then separated into fast-spiking interneurons or pyramidal cells through a specific criteria.

1.5. Spike detection and sorting

As stated out above, two main neuron types take role in the generation of SPW-R, pyramidal cells and fast-spiking interneurons (Schlingloff et al., 2014). Tetrode recording of CA1 and CA3 pyramidal cell layer were used in this thesis to record the SPW-R oscillation and also the firing of single cells (called units). Firing of a unit is called a ‘spike.’ It is also known that cell firing is coupled to SPW-R (Both et al., 2008) so to analyze single cell firing, they must be separated from the SPW-R which is done by applying a band pass filter on the raw data to isolate the spikes of various units.

Following this filtering, an amplitude threshold is set to distinguish spikes from background noise. Thereafter, specific features of all the spikes that were detected are extracted and the features that best separate spikes into different units are fed into a clustering algorithm that sorts the spikes into various units (Chaure et al., 2018) (Figure 5). Following this automated process the experimenter observes the units that were generated and further sorts the spikes based on spike shape and other parameters explained in the methods section. The final aim is to separate the spikes with as much clarity into various units while reducing the background noise to a minimum.

Following spike sorting, the resulting units were separated into fast-spiking interneuron and slow-spiking pyramidal cells using four parameters related to spike firing and spike shape that

were established to be the most reliable in distinguishing between these two types of neurons (Sirota et al., 2008; English et al., 2017). The method of separation and the four parameters is explained further in the methods section.

Using tetrode recordings improves the process of spike sorting. Tetrodes are comprised of four electrodes in close proximity, which leads to recording of a single neuron from four different sites (Quiroga, 2012). This feature of the tetrodes has been shown to increase the single cell yield of the recordings while also achieving more accurate spike sorting since an ambiguous sorting of the spikes from one channel can be solved by the signal from the other three channels (Quiroga, 2012). So, to put it simply, in a tetrode recording, each unit has a distinct spike shape on four channels rather than just one, so spikes can be sorted into units with much more certainty than they can be from single channel recordings.

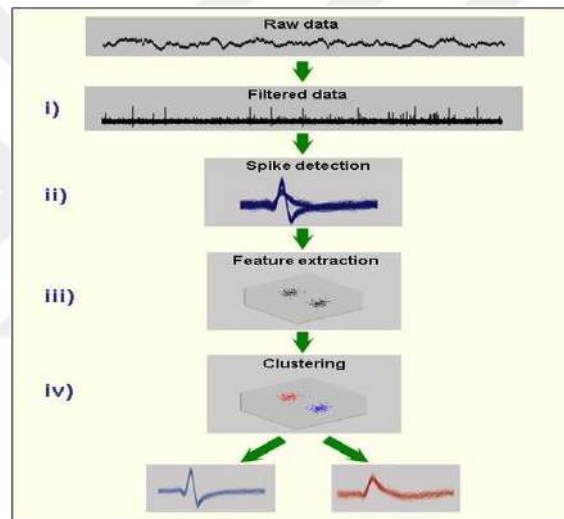


Figure 5: The steps of spike sorting. **i)** High pass filtered recording from the human right entorhinal cortex. **ii)** Spikes are detected after an amplitude threshold is set. **iii)** Spike shape features that best separate spikes into units are determined. **iv)** Spikes are clustered into various units. Figure adapted from (Quiroga et al., 2004).

1.6. Axon initial segment plasticity

Axon Initial Segment (AIS) is a special structure of the axon that is usually placed in the unmyelinated proximal axon, close to the cell soma, and around 20 to 60 μm in length for the hippocampal neurons (Thome et al., 2014). Due to the high density of voltage gated ion-channels on the membrane of the AIS mainly for sodium, potassium and calcium, it functions as the initiator of the action potential (Huang and Rasband, 2018). The backbone of the AIS structure is the cytoskeletal protein β 4-spectrin which binds to actin filaments and to Ankyrin

G, which then bind to voltage gated ion-channels and clusters these channels onto the AIS membrane (Huang and Rasband, 2018; Leterrier et al., 2015; Zhong et al., 2014). The actin/ β 4-spectrin/Ankyrin-G complex forms a scaffold which itself acts as a diffusion barrier and this barrier separates the axon from the cell soma, maintaining neuronal polarity (Huang and Rasband, 2018; Leterrier et al., 2015).

After chronic depolarization, the AIS of a neuron either moves away from the cell soma (Grubb and Burrone, 2010b), or shortens (Evans et al., 2015), which decreases the excitability of the neuron (Yamada and Kuba, 2016). In the event of a prolonged period without excitation, such as sensory deprivation, the AIS lengthens, increasing the excitability of the neuron (Gutzmann et al., 2014, Kuba et al., 2010) (Figure 6). In both cases the AIS responds to a change in excitation by adjusting the excitability of the neuron, so that a homeostatic neuronal output of a neuronal network can be preserved (Huang and Rasband, 2018). In one experiment, optogenetic stimulation of the granule cells of the dentate gyrus (DG) lead to a shortening of the AIS which was completely reversible in just 3 hours (Evans et al., 2015).

Little is known about the effect of mild or strong OGD on the AIS of the neurons of the hippocampal region so investigating this would be a novel endeavor. In this thesis, the slices that had undergone mild or 20-minute OGD, or 21-hour incubation in the UV-modified interface chamber were stained for β 4-spectrin along with their respective control slices. Then these slices were imaged under a confocal microscope and analyzed for number of AIS and length of AIS to assess whether these procedures lead to a significant difference between experiment and control slices.

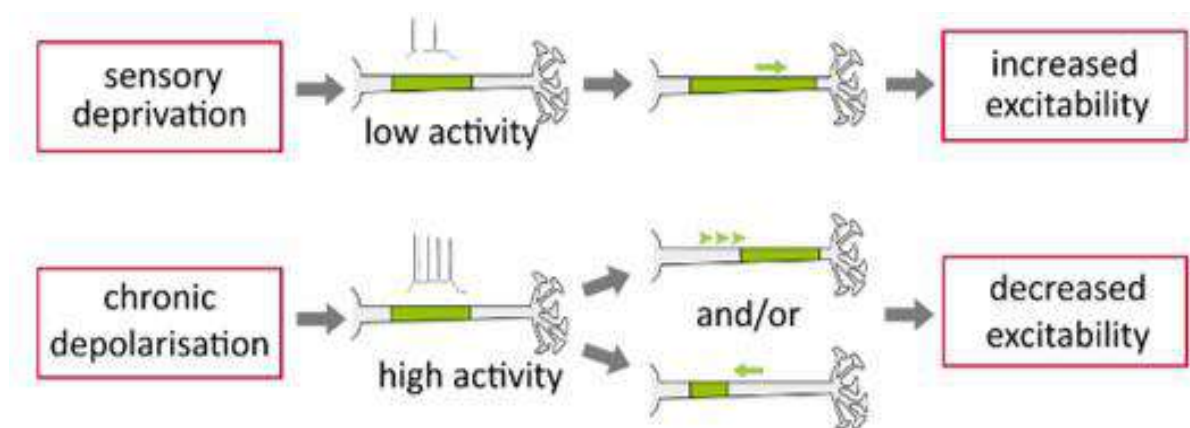


Figure 6: Visualization of AIS plasticity in response to different stimuli. Following sensual deprivation, AIS lengthens, following chronic depolarization, the AIS moves away from the soma or shortens. Figure adapted from (Jamann et al., 2018).

1.7. Aim of the thesis

The aim of this thesis was to study the neuronal recovery of old mice hippocampal slices from mild OGD and to compare the effects of mild and severe OGD on AIS morphology as well as to find a method to extend the experimental window of OGD modelling *in vitro* by extending the viability of slices. It has been shown before that mature adult mice (aged 33-66 weeks) hippocampal neurons regain their membrane potential and regain the ability to fire action potentials (Hefter et al., 2016). Mild OGD experiments on young adult mice (aged post-natal day 60-90) showed a partial recovery of SPW-R and unit firing (Grube et al., manuscript in print). However, no mild OGD experiment was performed on old mice, which is a promising project since aging is known to be the leading risk factor of ischemic stroke (Yousufuddin and Young, 2019).

Through the help of tetrode recordings and the Wave_clus algorithms, the spikes detected from these recordings can be sorted into units (Chaure et al., 2018) and these units can be separated into fast-spiking interneurons and pyramidal cells (Sirota et al., 2008; English et al., 2017). This is also valuable since it has been shown that interneuron are more vulnerable to mild metabolic stress (Povysheva et al., 2019, Elzoheiry et al., 2020). OGD until SD was performed on old mice (aged 75 -104 weeks), and the recovery of SPW-R and unit activity of the CA1 and CA3 regions were studied as well as the differential effect OGD might have on fast-spiking interneurons and pyramidal cells. The effect of mild OGD on AIS morphology was also analyzed.

To extend the viability of acute brain slices a modified Hass-type interface chamber with UV light was used along with low concentrations of penicillin and streptomycin (inspired from Buskila et al., 2014). The effect of 21-hour incubation in the modified interface chamber on the SPW-R of the CA1 and CA3 regions of young adult mice (post-natal day 60-70) was studied as well as the AIS morphology of these slices following 21-hour incubation.

A stronger OGD experiment was performed on hippocampal slices of adolescent mice (postnatal day 28-40) in which slices underwent OGD for 20 minutes regardless of when SD occurred and were fixated in 1 % Paraformaldehyde (PFA) either immediately after, one hour post OGD, or four hours post OGD. The effect of this 20-minute OGD insult on the number of AIS of the CA1 region of the three groups of slices were analyzed and compared.

2. MATERIALS AND METHODS

2.1. Animals and Tissue Handling

All experiments were performed in accordance with state law and institutional guidelines. For the mild Oxygen Glucose Deprivation (OGD) experiments, eleven mice expressing tdTomato in PV+ interneurons (B6-129P2-Pvalb^{tm1} (cre)Arbr/Uhg; B6-Gt(ROSA)-26Sortm14(CAG)Hze; below abbreviated as PV-tdTomato) that were bred by the Draguhn Lab of both sexes were sacrificed on postnatal week 75 to 104. For the 21-hour ultraviolet-penicillin-streptomycin (UVPS) experiments, two male Wild type (WT) C57BL/6 mice obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) were sacrificed on postnatal day 60-70 and for the 20-minute OGD experiment, 8 male Wild type (WT) C57BL/6 mice obtained from Janvier Laboratories (Le Genest-Saint-Isle, France) were sacrificed on P 28-40. The sacrifice was achieved by deeply anesthetizing the mice using CO₂ and decapitating the mice following the loss of righting reflex except for the mice that were used in the 20-minute OGD experiment which were deeply anesthetized using Isoflurane Baxter.

Following decapitation the head was immediately transferred into ice-cold Artificial Cerebrospinal Fluid (ACSF) and the brain was swiftly removed from the skull, trimmed and glued onto a cutting platform, surrounded by ACSF at 2-6°C saturated with 95 % O₂ / 5 % CO₂ (carbogen). Composition of ACSF was as follows: (in mM): 124 NaCl, 3.0 KCl, 1.8 MgSO₄, 1.6 CaCl₂, 10 glucose, 1.25 NaH₂PO₄ and 26 NaHCO₃, pH 7.4. Horizontal slices were cut to 450 µm thickness using a Leica V1200s vibratome (Leica Microsystems, Wetzlar, Germany). Slices containing the ventral to mid portion of the hippocampus were then transferred to a Hass-type interface chamber and left to recover for at least 2 hours (3 hours for the mice used in the mild OGD experiment, due to their old age) at 32-34°C while they were perfused with oxygen-saturated ACSF with a flow rate of 1.5-2ml/minute.

2.2. Mild OGD (OGD until SD) of old mice

Before the start of mild OGD electrophysiological data was recorded from experiment and control slices for 20 minutes using tetrodes that were placed at the CA1 and CA3 stratum pyramidale (pyramidal cell layer) after the hippocampal slices from mice aged 75-104 weeks, were allowed to recover for 3 hours. For the OGD slices a glass electrode was also placed on the CA1 stratum pyramidale, further away from the CA1 tetrode and closer to the subiculum, in order to record the LFP so that the SD induced by hypoxia-ischemia can be observed. To model hypoxia-ischemia (HI) the perfusion was switched to an equimolar ACSF solution

containing mannitol (Mannitol-ACSF), instead of glucose, saturated with an oxygen-free gas mixture of 95 % N₂ and 5 % CO₂. As soon as spreading depression was observed in the LFP recording, perfusion was switched back to standard ACSF saturated with carbogen. After this reperfusion with standard ACSF, the recovery of the slices from mild OGD was recorded via tetrodes at CA1 and CA3 stratum pyramidale for one hour. Both experiment and control slices were then left to remain in their respective interface chambers and 20 minute tetrode recordings were again taken from both slices at the CA1 and CA3 stratum pyramidale from three hours 40 minutes to four hours after the SD occurred in the experiment slice. After this 20 minute tetrode recording, both experiment and control slices were put into a 1 % Paraformaldehyde solution (PFA) overnight for fixation and the following day the slices were transferred to Phosphate-buffered saline (PBS) solutions for further immune-staining which will be explained in the Histology section.

2.3. 20-minute OGD

After 2 hours of incubation of experiment and control hippocampal slices from adolescent mice aged postnatal day 28-40, in their respective interface chambers, a glass electrode recording was made to record the LFP at the CA1 stratum pyramidale to check whether the slices had healthy SPW-R activity. After the confirmation of healthy activity in both experiment and control slices, 20 minute of OGD was induced by switching to a perfusion of Mannitol-ACSF saturated with an oxygen free gas mixture of 95 % N₂ and 5 % CO₂. After 20 minutes of OGD, the perfusion was switched back to standard ACSF, saturated with carbogen. Some experiment and control slice pairs were put into 1 % PFA immediately after the 20-minute OGD, some pairs were put into PFA after a recovery period of one hour and some were put into 1 % PFA after a recovery period of four hours. Before the one hour and four hour recovery slice pairs were put into 1 % PFA, a glass electrode recording was taken from the CA1 subiculum pyramidale of both experiment and control slices and the slice pair was not taken into account if after the recovery period the control slice showed no SPW-R activity. The experiment slice was allowed to show no SPW-R activity since that slice had undergone 20- minute OGD. After the slices remained in 1 % PFA overnight, they were put into PBS for further immuno-staining.

2.4. 21-hour incubation with ultraviolet-penicillin-streptomycin (UVPS)

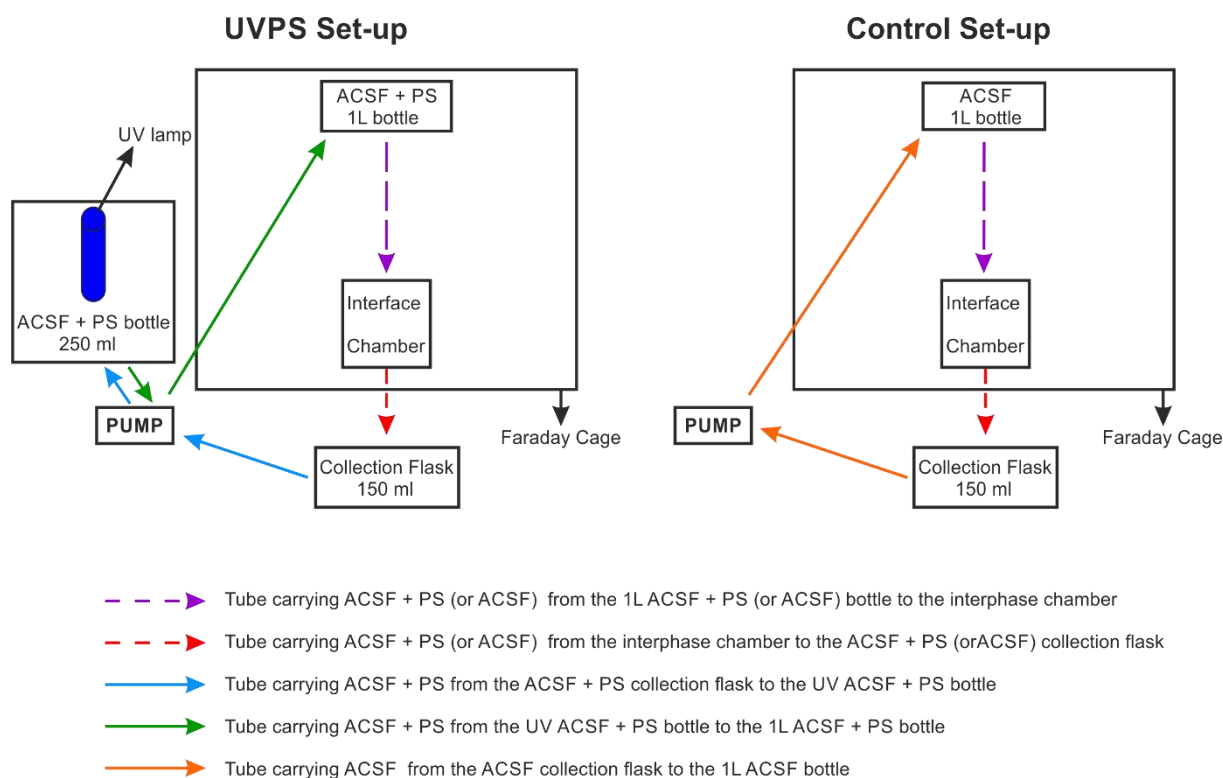
To study whether hippocampal slices can survive overnight incubation in a Hass-type interface chamber, ultraviolet light and an ACSF solution containing Penicillin and Streptomycin (referred to as ACSF + PS from now on) were used in experiments on hippocampal slices from young adult mice aged postnatal day 60-70. Concentration of antibiotics used in ACSF + PS was as follows: 0.042 Penicillin G sodium, 0.043 Streptomycin. The purpose of this ultraviolet plus ACSF + PS (UVPS) method was to keep the ACSF and the interface chamber as free of bacteria as possible, modified from a previous paper which used a submerged method and UV light to keep 300 μm thick hippocampal slices alive for up to 36 hours. (Buskila et al., 2014) In this paper, researchers used a special chamber called 'Brincubator' to store brain slices in a submerged environment and run the ACSF through a special UV chamber to decrease growth of bacteria. (Buskila et al., 2014)

The UV method used here is adapted from the 'Brincubator' but modified to keep the slices incubated on a Hass-type interface chamber so that various experiment such as OGD can be performed on the slices with an overnight recovery period and tetrode recordings can be taken. Penicillin and streptomycin was added into the ACSF along with UV light to decrease the bacteria growth within the tube walls that ACSF passes through and the filter paper that the acute brain slices lay on while inside the interface chamber. To test whether the UVPS method is beneficial to the acute brain slices, control slices were put into another interface chamber where ACSF is cycled with the use of a pump directly from the collection flask to the 1-liter ACSF bottle without the use of UV light or penicillin and streptomycin. Below the UVPS and control set-ups can be seen. (Figure 1)

As done in the Buskila et al. (2014) paper, the UV lamp was connected to a timer that turned it on and off at random intervals to prevent the ACSF solution from overheating due to the UV light emission. The timer turned the UV light ON randomly for 10 to 26 minutes and turned the UV light OFF randomly for 26 to 42 minutes. The timer used was an EMT757 type timer (230V-50Hz, 8.7A) obtained from Everflourish (Friedrichsthal, Germany). The UV lamp was a 7 watts waterproof UV lamp normally used to sterilize aquariums, obtained from Roxin. After each experiment, the UV lamp was cleaned with distilled water to prevent salt crystals from forming on the glass of the lamp due to the ACSF + PS solution.

The 450 μm thick hippocampal slices were put into separate Haas-type interface chambers with UVPS and the control set up. (Figure 7) After 2 hours of recovery time baseline tetrode

recording for 10 minutes were taken from CA1 and CA3 stratum pyramidale of both experiment and control slices. The slices were left in the interface chamber overnight for a total of 21 hours. Afterwards, 10 minutes tetrode recordings from CA1 and CA3 stratum pyramidale of both experiment and control slices. Then both experiment and control slices were put into 1 % PFA overnight and later put on PBS for further immune-staining.



Dashed lines show tubes carrying ACSF with the use of gravity, while filled in lines show tubes carrying ACSF with the use of the pump.

Figure 7: UVPS and control set-ups that were used in the UVPS experiment. The UV lamp is outside of the Faraday Cage, to reduce the electrical noise that it produces. To reduce the noise even further, a silver wire was put inside 250 ml bottle containing the UV lamp, this wire was connected to a cable and was grounded (cable and wire not shown on the figure).

2.5. Electrophysiological Recordings

2.5.1. Glass Electrode Recordings

For the mild and 20-minute OGD experiments, glass microelectrodes were made which had tip diameters larger than $5\mu\text{m}$ and were filled with ACSF before use. A silver wire was placed inside the ACSF filled glass electrodes which carried the LFP signal to an EXT 10–2F amplifier (npi electronic, Tamm, Germany) that amplified it $\times 100$. This signal was then low-pass filtered at 3,000 Hz, and digitized at 20 kHz for off-line analysis using the 1401 interface and Spike 2 data acquisition program (CED, Camebridge, UK). In the mild OGD experiments, the glass

electrode recordings were used to detect the spreading depression and in the 20-minute OGD experiments, these recordings were used to check whether the slices had healthy SPW-R activity during baseline and whether the control slices had healthy SPW-R activity just before the experiment-control slice pairs were put into 1 % PFA for fixation.

2.5.2. Tetraode Recordings

LFP and single unit discharges (spikes) were recorded with two tetrodes placed in CA1 and CA3 stratum pyramidale for the mild OGD experiments. The tetrodes were made of four twisted 12.5µm-diameter tungsten wires (California Fine Wire, Grover Beach, USA) and connected to a DPA-2FX amplifier (npi electronics, Tamm, Germany). Signals were amplified 100 times using a low-pass filtered at 10 kHz, high pass filtered at 0.3 Hz and digitized at 20 kHz for off-line analysis using the 1401 interface and Spike-2 data acquisition program (CED, Cambridge, UK).

2.6. SPW-R Analysis

LFP recordings were analyzed using custom-written MATLAB routines. Briefly, SPW-R were detected as local maxima in 10-x downsampled, < 50 Hz lowpassed signal. Ripples were detected as local maxima in 10-x downsampled, 100 – 300 Hz bandpass-filtered signal. Ripple properties were analyzed using Morlet wavelet transformation of time windows starting 33 ms before and ending 67 ms after the peak of each SPW. Maximum power between 140 – 360 Hz was detected; the corresponding frequency was defined as the ripple frequency. Ripple energy was defined as the integral of values above 0.5 of maximal power in this frequency range. For the mild OGD analysis, tetraode recordings during baseline condition with Ripple Energy less than 2.5 a.u. and SPW frequency less than 1 Hz were deemed unfit and discarded.

2.7. Spike Sorting and Unit Properties

Spike detection and sorting of the spikes into units were performed using the Wave_clus program (Quiroga et al., 2004; Chaure et al., 2018). As mentioned in the Introduction, this method is used to detect the firing of certain units (spikes) by isolating them from the background noise and sorting them into separate, well-defined units based on several properties (amplitude, trough-to-right-peak latency etc.). To achieve spike detection the signal was band-pass filtered (300 – 3000 Hz) and an amplitude threshold was set based on the estimated standard deviation of the background noise. Then, wavelet transforms for the detected spikes were performed and a number of wavelet coefficients that were best suited for separating the

spikes were automatically selected based on their deviation from normality. Based on these wavelet coefficients superparamagnetic clustering, a simulation of physical interactions of data points and their K- nearest neighbors, was performed, and the resulting clusters saved.

Most spike sorting algorithms used are semi-automatic, meaning that an algorithm performs the initial clustering. This clustering is fed into a graphic interface where the experimenter can work further on the clusters (Harris et al., 2016) and this was also done in this thesis. After the initial sorting, the experimenter used the Wave_clus graphic interface (GUI) to sort the spikes further based on spike shape, autocorrelogram to separate or combine units as well as discard some units as noise. (Figure 8)

The Wave_clus GUI provides the experimenter with cluster size and temperature threshold settings which can be used to adjust acceptable cluster sizes or the degree of unit separation (temperature) (Chaure et al., 2018) (Figure 8). Increasing the temperature and decreasing cluster size, leads to increased number of units and vice-versa (Chaure et al., 2018) The experimenter used these thresholds to achieve the clearest sorting of the spikes into units with the least amount of noise. (Figure 8)

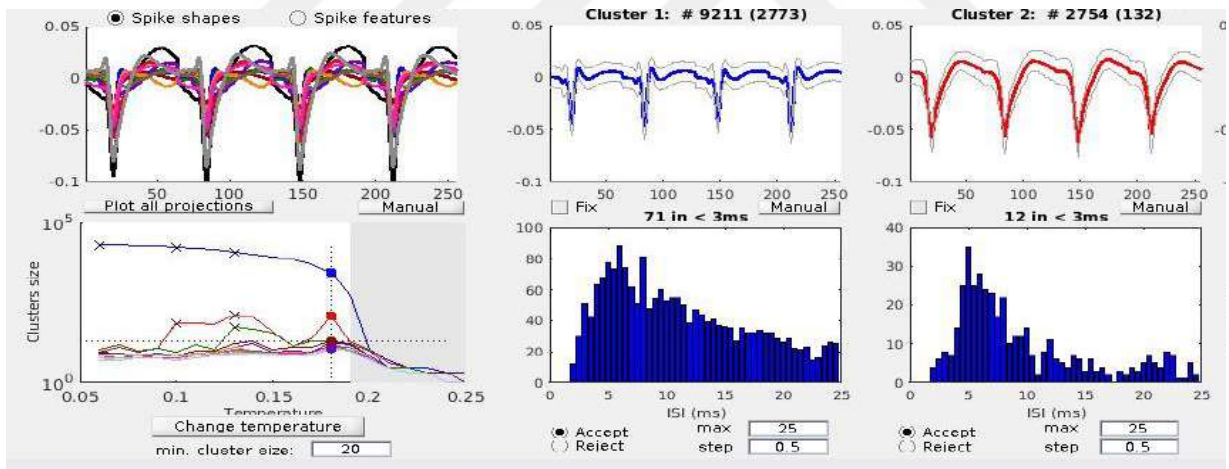


Figure 8: Wave_clus GUI example. Top column shows spike shapes of two clusters, bottom column shows the cluster-size and heat map used to separate the spikes (bottom left) as well as autocorrelograms that were used to determine whether the spikes were contaminated with noise (bottom middle and right).

After the units were detected by their specific waveforms (spike shapes) by the wave-clus algorithm (Quiroga et al., 2004; Chaure et al., 2018), certain variables that characterize the spike shapes were analyzed. Figure 9 shows specific marks and arrows to assist the reader in the explanation of these specific variables that are measured from the unit's waveform.

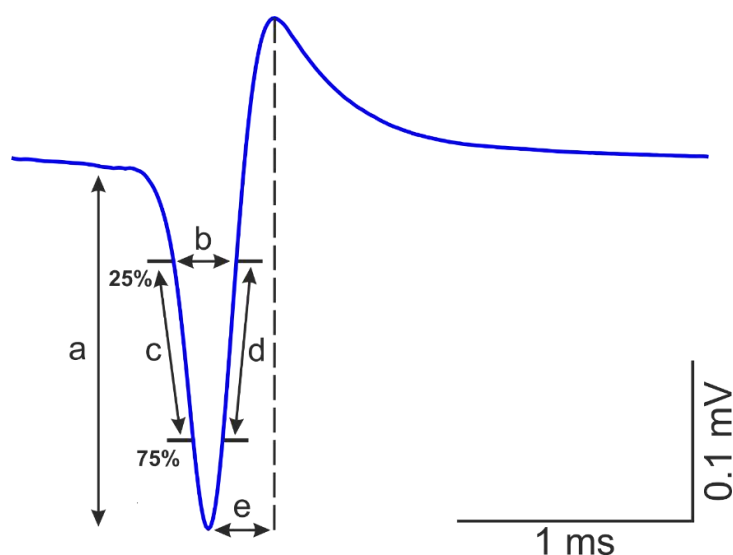


Figure 9: Features used to evaluate the waveform of a spike. Certain lines and arrows are put in to help reader understand some of the variables that are obtained by measuring certain aspects of the unit's waveform.

Amplitude: Amplitude of a unit's spike is the maximum deviation from the resting potential in mV that the unit reaches, in our tetrode recordings this is usually a negative value since the spikes are recorded as negative deviations of the field potential. However, this is then converted to positive values since the sign is not important. In figure 9, the arrow labeled 'a' indicates the amplitude, the arrow's length in millivolts would be the amplitude of the unit. As the spike reaches the maximum amplitude and comes back, of course some time passes, which is used to explain the next variable of 75 % amplitude width (Width75).

75 % amplitude width (Width75): 75 % amplitude width is measured as the time it takes for an individual spike to go from 25 % of the amplitude to the maximum amplitude and come back to 25 % of its amplitude. In other words, it is the width of the spike taken not encompassing the entire amplitude but 75 % of the amplitude. It is in milliseconds. In figure 9, horizontal lines are drawn on the unit to signify the point's on the unit waveform that are 25 % and 75 % of the amplitude. The arrow labeled 'b' indicates the 75 % amplitude width measurement, the length of this arrow in milliseconds would be the 75 % amplitude width of the unit.

Normalized rate of fall (Fall-Norm): Normalized rate of fall is the velocity of the spike to go from 25 % of the amplitude to 75 % of the amplitude in mV per second, normalized to the amplitude of the spike by dividing the velocity to the amplitude value. Therefore, the unit of this variable is 1/seconds. In figure 9, the arrow labeled 'c' follows the unit waveform as it falls from 25 % of the amplitude to 75 % of the amplitude, as can be seen the arrow moves both

vertically (in millivolts) and horizontally (in milliseconds), normalized rate of fall is calculated by dividing the distance the arrow covers in millivolts by the distance it covers in milliseconds and normalizing this value by the amplitude of the unit.

Normalized rate of rise (Rise-Norm): Normalized rate of rise is calculated in the same manner as normalized rate of fall (fall-norm), but this time the velocity of the spike to go from 75 % of the amplitude to 25 % of the amplitude is normalized to the amplitude of the spike. In figure 9, the arrow labeled 'd' follow the unit waveform as it rises from 75 % of the amplitude to 25 % of the amplitude. As normalized rate of fall, normalized rate of rise is calculated by dividing the distance the arrow covers in millivolts by the distance it covers in milliseconds and normalizing this value by the amplitude of the unit. So the variables of normalized rate of fall and normalized rate of rise indicate how fast a unit falls and rises again, between 25 % and 75 % of the amplitude. The unit of Rise-Norm is also 1/seconds.

Trough-to right –peak latency (tRightPeak): In each cell firing, there is a small off-shoot of the resting potential as the cell goes back to its resting potential. In the waveform of the unit, this is seen as a wave on the right side of the amplitude that is smaller than the amplitude and is in the opposite direction as the amplitude wave. Trough-to-right-peak latency, is the time between the peak of this wave and the trough of the amplitude wave i.e. maximum amplitude. It is in milliseconds. In figure 9, the dashed line indicates the peak of the wave on the right side of the amplitude, and the arrow labeled 'e' indicates tRightPeak. The distance the arrow covers in milliseconds would be the trough-to-right-peak latency of the unit shown in figure 9.

There are also other variables that connect unit activity to the overall network oscillation. They are calculated as follows:

Frequency: This is a measure of how many times per second a specific unit fires. The unit of this variable is Hz or 1/s.

Sharp wave Specificity: This is the measure of specificity of spike times to SPW-R time-frames, in other words, a measure how specifically a unit fires within SPW-R time frames. It is calculates as $(f_{\text{inside}} - f_{\text{outside}})/(f_{\text{inside}} + f_{\text{outside}})$ where f_{inside} refers to the number of spikes of a unit that fire within an SPW-R time frame and f_{outside} refers to the number of spikes of a unit that fire outside of an SPW-R time frame. A value of 1 indicates that a given unit fired only within SPW-R and a value of -1 indicates that a given unit fires only outside of SPW-R.

Ripple Phase (or Median Ripple Phase): If you think of a ripple oscillation as a sine wave, one can assign each spike of a unit a degree on the ripple sine wave where it fired, ranging from -180° to 180° . Then a cumulative histogram can be plotted for each unit that plots the cumulative number of spikes that fired from -180° to 180° covering the entire ripple sine wave. A ripple phase for a unit can then be assigned to the degree where 50 % of the spikes of the unit fired on the cumulative histogram graph. This is the median ripple phase where units fired. The unit of this variable is degrees ($^\circ$).

Ripple Phase Specificity: Just as the ripple phase, this variable is calculated by thinking of the ripple oscillation as a sine wave and plotting a cumulative histogram of total spikes of a unit that fired from -180° to 180° . If a unit fired without any phase specificity, the cumulative histogram line would be linear since the increase of spikes through the degrees would be uniform. In this case, the difference in degrees between the degree where 75 % of the spikes of the unit fired and the degree where 25 % of the spikes of the unit fired would be 180. That value is the maximum difference this range can have at no specificity. So if we call this difference drange, then specificity can be calculated as $(180 - (\text{drange})) / 180$. If a unit has phase specificity of 1, this means that the unit fires only at a certain degree and that in the cumulative histogram plot, the drange value is 0, meaning there is no difference between the degree where 75 % of the spikes fired and the degree where 25 % of the spikes fired, hence $(180 - 0) / 180 = 1$.

For the variables of frequency, sharp wave specificity, ripple phase and ripple phase specificity, multi-unit activities (MUA) are also shown in the results section. These are calculated without separating the units as the name suggests, so the units are not sorted. For example, MUA frequency shows the spike firing per second of all units, unsorted. Thus, MUA frequency of 7 would indicate that 7 spikes belonging to various units are fired per second.

2.8. Separation of Units into different classes

Units were further separated into pyramidal cell (PYR) and fast-spiking interneuron (FSI) classes using four physiological parameters that have been shown to reliably distinguish these cell types (Sirota et al., 2008; English et al., 2017). For this, the K-Means algorithm was run on four z- score normalized dimensions: $\log_{10}$ Inter-Spike Interval (log of ISI) mode, log of ISI median, burst percentage (defined as number of ISI < 8 ms divided by number of all ISI * 100), and trough-to-right-peak latency (tRightPeak). There were some units that could not have been separated into either cell type following the K-Means algorithm, and they were put into a third class labeled as MIX, a mixture of PYR and FSI units that weren't sorted. So the units were

divided into 3 classes, Pyramidal Cell (PYR), fast-spiking Interneurons (FSI) and MIX. Below log of ISI and Burst Percentage variables are defined. Trough-to-right-peak latency (tRightPeak) was already defined in the previous section.

Log₁₀ of the inter-spike interval (log of ISI): Inter-spike interval is the time between spikes of a given unit in milliseconds and log of ISI is the logarithm of this value. So a value of 1 would correspond to 10 milliseconds, 2 would correspond to 100 milliseconds and so forth.

Burst percentage: If the inter-spike interval (ISI) between two spikes is less than 8 milliseconds than the spikes are considered to have fired in a burst. This variable is the percentage of spikes of a given unit with that fire with an ISI less than 8 milliseconds (number of ISI < 8 ms divided by number of all ISI * 100).

In the figure below example traces of PYR and FSI unit types are shown to make clear the differences in the waveform of these units.

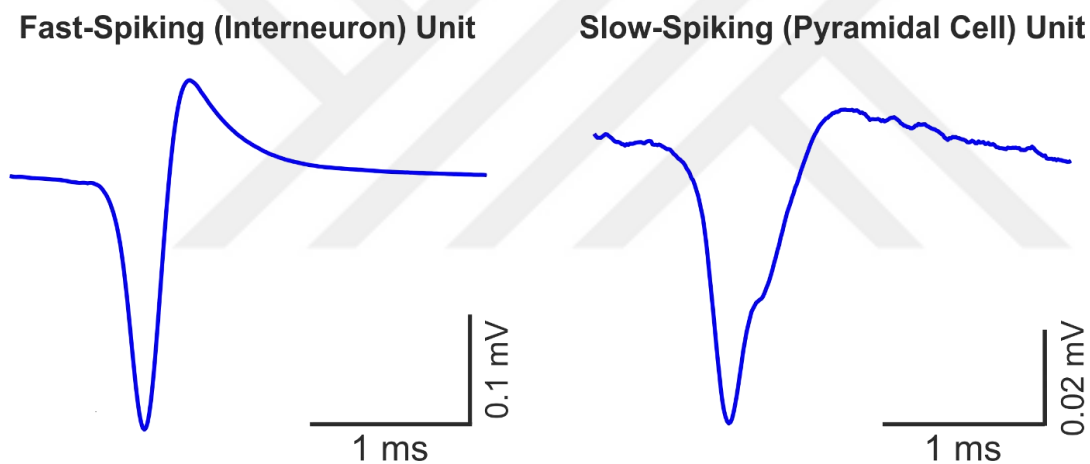


Figure 10: Fast-spiking and slow-spiking unit traces. As can be seen, the Fast-Spiking unit has a smaller trough-to-right-peak latency (tRightPeak) than the Pyramidal Cell unit, which is one of the four physiological parameters that were used to distinguish units (Sirota et al., 2008; English et al., 2017).

2.9. Histology

2.9.1. Staining of slices following mild OGD

After recording, slices were fixated in 1 % PFA for overnight and afterwards stored in PBS at 4°C for further staining for NeuN (red channel), AIS (β4-Spectrin) (dark red channel) and DAPI (blue channel). Slices were washed three times in PBS for 10 minutes and blocked with 5 % normal goat serum/ 0.3 % TritonX-100 in PBS for 2 hours. Afterwards, they were washed again in PBS three times for 10 minutes and incubated with mouse-anti-NeuN (1:500, Synaptic

Systems, Göttingen, Germany) and rabbit anti- β 4-Spectrin (1:1000, Swant, Marly, Switzerland) primary antibodies in 1 % normal goat serum/ 0.2 % TritonX-100 in PBS for 18 hours at room temperature. Upon washing with PBS three times for 10 minutes, slices were incubated with Alexa 546 goat-anti-mouse (1:1000, Thermo Fisher Scientific, Waltham, USA), and Alexa 647 goat-anti-rabbit (1:1000, Thermo Fisher Scientific) secondary antibodies in 1 % normal goat serum/ 0.2 % TritonX-100 in PBS for 2 hours at room temperature under light protection. Slices were then again washed with PBS three times for 10 minutes and mounted onto slides with Fluoroshield (Sigma-Aldrich, St. 4 Louis, USA), containing DAPI, for nuclear staining.

2.9.2. Staining of slices following 20-minute OGD

After recording, slices were fixated in 1 % PFA for overnight and afterwards stored in PBS at 4°C for further staining for, AIS (β 4-Spectrin) (dark red channel) and DAPI (blue channel). Slices were washed three times in PBS for 10 minutes and blocked with 5 % normal goat serum/ 0.3 % TritonX-100 in PBS for 2 hours. Afterwards, they were washed again in PBS three times for 10 minutes and incubated with rabbit-anti- β 4-Spectrin (1:1000, Swant, Marly, Switzerland) primary antibody in 1% normal goat serum/ 0.2 % TritonX-100 in PBS for 18 hours at room temperature. Upon washing with PBS three times for 10 minutes, slices were incubated with Alexa 647 goat-anti-rabbit (1:1000, Thermo Fisher Scientific) secondary antibodies in 1% normal goat serum/ 0.2 % TritonX-100 in PBS for 2 hours at room temperature under light protection. Slices were then again washed with PBS three times for 10 minutes and mounted onto slides with Fluoroshield (Sigma-Aldrich, St. 4 Louis, USA), containing DAPI, for nuclear staining.

2.9.3. Staining of slices following 21-hour incubation in the UVPS set-up

After recording, slices were fixated in 1 % PFA for overnight and afterwards stored in PBS at 4°C for further staining for NeuN (green channel) ,AIS (β 4-Spectrin) (dark red channel), Parvalbumin (PV) (red channel) and DAPI (blue channel). Slices were washed three times in PBS for 10 minutes and blocked with 5 % normal goat serum/ 0.3 % TritonX-100 in PBS for 2 hours. Afterwards, they were washed again in PBS three times for 10 minutes and incubated with chicken-anti-NeuN (1:500, Synaptic Systems, Göttingen, Germany), mouse-anti-PV (1:1000, Swant, Marly, Switzerland) and rabbit-anti- β 4-Spectrin primary antibodies in 1 % normal goat serum/ 0.2 % TritonX-100 in PBS for 18 hours at room temperature. Upon washing with PBS three times for 10 minutes, slices were incubated with Alexa 488 goat-anti-chicken (1:1000, Thermo Fisher Scientific, Waltham, USA), Cy3 goat-anti-mouse (1:500, Dianova,

Hamburg, Germany) and Alexa 647 goat-anti-rabbit (1:1000, Thermo Fisher Scientific) secondary antibodies in 1 % normal goat serum/ 0.2 % TritonX-100 in PBS for 2 hours at room temperature under light protection. Slices were then again washed with PBS three times for 10 minutes and mounted onto slides with Fluoroshield (Sigma-Aldrich, St. 4 Louis, USA), containing DAPI, for nuclear staining.

2.9.4. Imaging and AIS analysis

Fluorescence images were taken on a Nikon A1R (Nikon Instruments Inc., Melville, USA) confocal microscope system using a Nikon Plan Apo λ 20x NA 0.7 objective. These images were analyzed using the Fiji Image J image processing software.

AIS analysis of slices following mild OGD

20x magnified z-stack images of the CA1, CA3 and DG regions of the OGD and control slices were taken that allows the experimenter to view deeper layers of the slice. For the AIS analysis, 10 AIS were selected at the pyramidal cell layer of the CA1 and CA3 regions and 10 AIS were selected for the hilus regions of the DG region of both OGD and control slices. Using the z-stack the beginning and end of these AIS were able to be determined and the length of these 10 AIS were measured in micrometers. Then the median length of these AIS for each region of each OGD and control slice was calculated using MATLAB and the OGD and control slices were compared.

For the AIS number analysis, a smaller region of the 20x magnified z-stack images were selected. For the CA1 and CA3 pyramidal cell layer, a $10011.68 \mu\text{m}^2$ square was selected for the z-stack layer which was $16.5 \pm 6 \mu\text{m}$ underneath the surface of the slice and for the hilus of the DG region, a $32444 \mu\text{m}^2$ square was selected for the z-stack layer which was $16.5 \pm 6 \mu\text{m}$ underneath the surface the slice. The AIS inside these selected regions were counted by the experimenter and compared statistically for OGD and control slices on MATLAB.

PV + INT AIS analysis was done by making a composite image of the PV + (red) and AIS (deep red) channels. For the 20x magnified z-stack images, it was not possible to reliably distinguish the AIS length of PV + INT from the AIS of the pyramidal cells in the pyramidal cell layers so for all regions the PV + INT outside the pyramidal cell layers were selected and the AIS of these interneurons were measured. Since in some slices there was no or very few PV + INT outside the pyramidal cell layers, all the PV + INT AIS were pulled together for the OGD and control slices and compared statistically on MATLAB.

To have a better comparison of the AIS length of the PV + INT and the percentages of PV + INT with AIS, 60x magnified z-stack images of the CA1 pyramidal cell layer of 8 OGD and 8 control slices were taken and the AIS of PV + INT were measured for these slices as well as the percentage of PV + INT with AIS. Again because numbers were low, the PV + INT AIS were pulled together for the OGD and control slices for AIS length comparison and to compare the percentage of PV + INT with AIS of the OGD and control slices statistically on MATLAB.

AIS analysis of slices following 20-minute OGD

For the AIS number analysis, a 10051.94 μm^2 square of the 60x magnified z-stack images of the CA1 pyramidal cell layer were selected for the z-stack layer which was $14.5 \pm 2 \mu\text{m}$ underneath the surface of the slice. The AIS inside these selected regions were counted by the experimenter and compared statistically for 20-minute OGD and control slices on MATLAB.

AIS analysis of slices following 21-hour incubation

60x magnified z-stack images of the CA1 pyramidal cell layer were taken that allows the experimenter to view deeper layers of the slice. For the AIS analysis, 20 AIS (or how many were visible) were selected at both UVPS and control slices. Using the z-stack the beginning and end of these AIS were able to be determined and the length of these 20 AIS were measured in micrometers. Then the median length of these AIS for each region of each UVPS and control slice was calculated using MATLAB and the UVPS and control slices were compared.

For the AIS number analysis, a 10051.94 μm^2 square of the 60x magnified z-stack images were selected for the z-stack layer which were $14.5 \pm 2 \mu\text{m}$ underneath the surface of the slice. The AIS inside these selected regions were counted by the experimenter and compared statistically for the UVPS and control slices on MATLAB.

PV + INT AIS analysis was done by making a composite image of the PV + (red) and AIS (deep red) channels. The AIS length of PV + INT were measured for these slices as well as the percentage of PV + INT with AIS. Because numbers per slices were low, the PV + INT AIS were pulled together for the UVPS and control slices for AIS length comparison and to compare the percentage of PV + INT with AIS of the UVPS and control slices statistically on MATLAB.

2.10. Statistical analysis

All statistical analysis and boxplots were performed on MATLAB, before comparing each set of variables, a Kolmogorov- Smirnov test was used to determine whether the values of each set had normal distribution. If both sets of variables had normal distribution, a two-sample student's t-test was used for independent variables and student's t-test was used for variables that were matched pairs. If one of the two sets of variables didn't have a normal distribution, a Wilcoxon rank-sum test was used for independent variables and a Wilcoxon signed-rank test was used for variables that were matched pairs.

Throughout this thesis, all data is represented as: median [25th, and 75th percentile] unless stated otherwise. All statistical analysis was performed on MATLAB. Null hypotheses were rejected at a significance level of $p = 0.05$.

3. RESULTS

3.1. Electrophysiological Results

3.1.1. Mild OGD reduces SPW-R frequency and unit coupling to SPW-R

Before the start of OGD (Oxygen-Glucose Deprivation), the slices were allowed to recover in the Haas-type interface chamber for 3 hours. Following this 20 minute tetrode recordings were taken from the CA1 and CA3 stratum pyramidale regions of the old mice hippocampus slices as a baseline for further comparisons. (Aged 75 weeks to 104 weeks post-natal). These recordings were taken from both the experiment slices that underwent mild OGD, and the control slices that stayed in the Haas-type interface chamber for the same duration as the experiment slices without mild OGD. In figure 1, 5 second and 100 millisecond example traces of these recordings taken from CA1 and CA3 can be seen. (Figure 1, A). In the 5 second recordings the characteristic slow oscillation of SPW-R's can be observed. In the 100 millisecond traces, a single SPW-R is visible, and ripples superimposed onto the sharp waves, as well unit firing can be seen. Unit firings are the downward spikes that are most visible in baseline CA3 SPW-R trace (Figure 1, A, bottom right).

In figure 1, the network oscillation properties of the baseline recordings can be seen. These are presented as boxplots, with CA1 and CA3 regions from left to right respectively, P-value for the statistical difference between baseline CA1 and CA3 regions were not calculated or shown since it is not the focus of this study. Rather, these baseline values are important to serve as comparison for the one hour post OGD (referred to as OGD 1h) and the four hour post OGD (referred to as OGD 4h) and four hour control conditions. For the boxplots in figure 1, experiment and control slice baseline recordings are pooled together since in pre-OGD the slices are expected to be in similar conditions and this gives a broader picture. The network oscillation values were as expected and fit within the parameters of the literature values during baseline conditions. After the MATLAB analysis of the network oscillation properties, there were 24 slices for CA1 and 25 slices for CA3, however, to assure that further analysis is performed on initially healthy slices, the data was filtered to include only the slices with sharp wave frequency larger than 1/s, and with Ripple Energy larger than 2.5 a.u. So 3 slices from CA1 and 1 slice from CA3 was eliminated from the baseline recordings. The corresponding 20 minute recordings taken at one hour and four hour post SD of these slices were also eliminated and statistical analysis was performed only on the remaining slices with healthy baseline SPW-R properties.

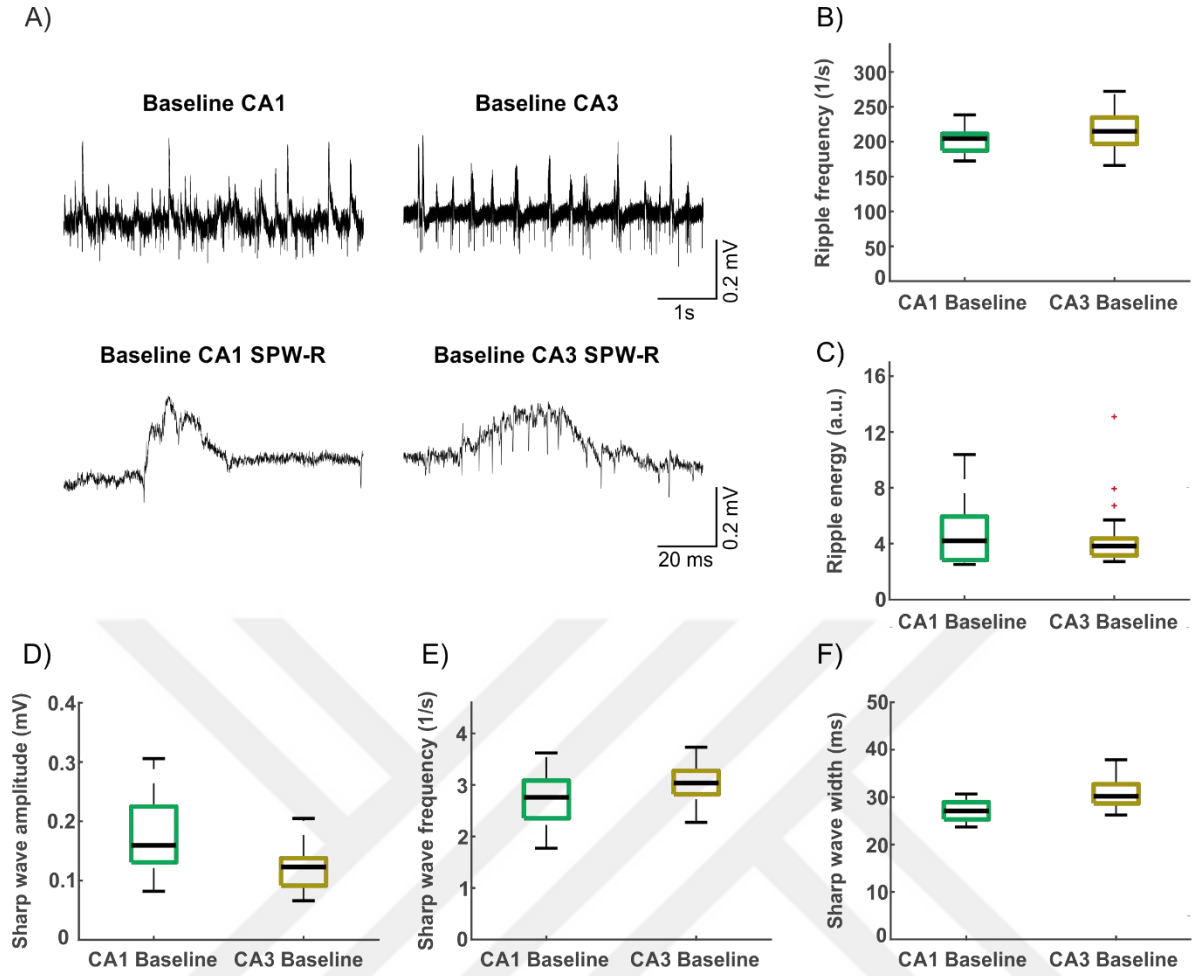


Figure 11: Example traces of tetrode recordings taken from CA1 and CA3 stratum pyramidale during baseline and baseline properties of SPW-R in CA1 and CA3. **A)** Top: Example traces of 5 second tetrode recordings taken from CA1 (left) and CA3 (right) at Baseline. Bottom: Example traces of 100 milliseconds tetrode recordings showing a single (SPW-R) taken from CA1 (left) and CA3 (right) at Baseline. **B-F)** Network oscillation property boxplots, in all boxplots, the network oscillation property shown from left to right are; CA1 Baseline (light green colored), CA3 Baseline (light brown colored). **B)** Ripple frequency (1/s). **C)** Ripple energy (arbitrary unit, a.u.). **D)** Sharp wave amplitude (mV). **E)** Sharp wave frequency (1/s). **F)** Sharp wave width boxplots (milliseconds, ms). $n_{CA1} = 21$ slices from 11 mice, $n_{CA3} = 24$ slices from the same 11 mice. (Baselines of OGD experiment slices and Control slices are pooled together.)

CA1 ripple frequency during baseline was 204.45/s (Hz) [187.22 211.31] and CA3 ripple frequency was 214.83/s (Hz) [196.75 234.62] (Figure 11, B). Ripple energy of CA1 during baseline was 4.19 a.u. [2.81 5.94] and ripple energy of CA3 was 3.82 a.u. [3.15 4.36] (Figure 11, C). Sharp wave amplitude of CA1 during baseline was 0.16 mV [0.13 0.22] and sharp wave amplitude of CA3 was 0.12 mV [0.09 0.14] (Figure 11, D). Sharp wave frequency of CA1 during baseline was 2.76/s (Hz) [2.35 3.08] and sharp wave frequency of CA3 was 3.04/s (Hz) [2.82 3.27] (Figure 11, E). Sharp wave width of CA1 during baseline was 27.08 milliseconds

(ms) [25.29 28.93] and sharp wave width of CA3 during baseline was 30.18 ms [28.65 32.73] (Figure 11, F)

In figures 12 and 13, multi-unit activity (MUA) and unit activity properties are shown for the baseline condition, respectively. In these figures the data is represented again as boxplots, with CA1 and CA3 region shown from left to right. These values also lie within the expected literature parameters. One thing worthy of note is the unit frequency boxplots, (Figure 13, B) where it can be seen that there are outlier units that fire more than ten times per second. There are also many units that fire with a lower frequency, this shows that there are both fast-spiking and slow-spiking units that were recorded at baseline condition and these units are then separated and analyzed to see how the specific unit properties change after mild OGD compared to control slices. Fast-spiking units are likely to be the inhibitory parvalbumin positive basket cells and the slow-spiking units are likely to be excitatory pyramidal cells.

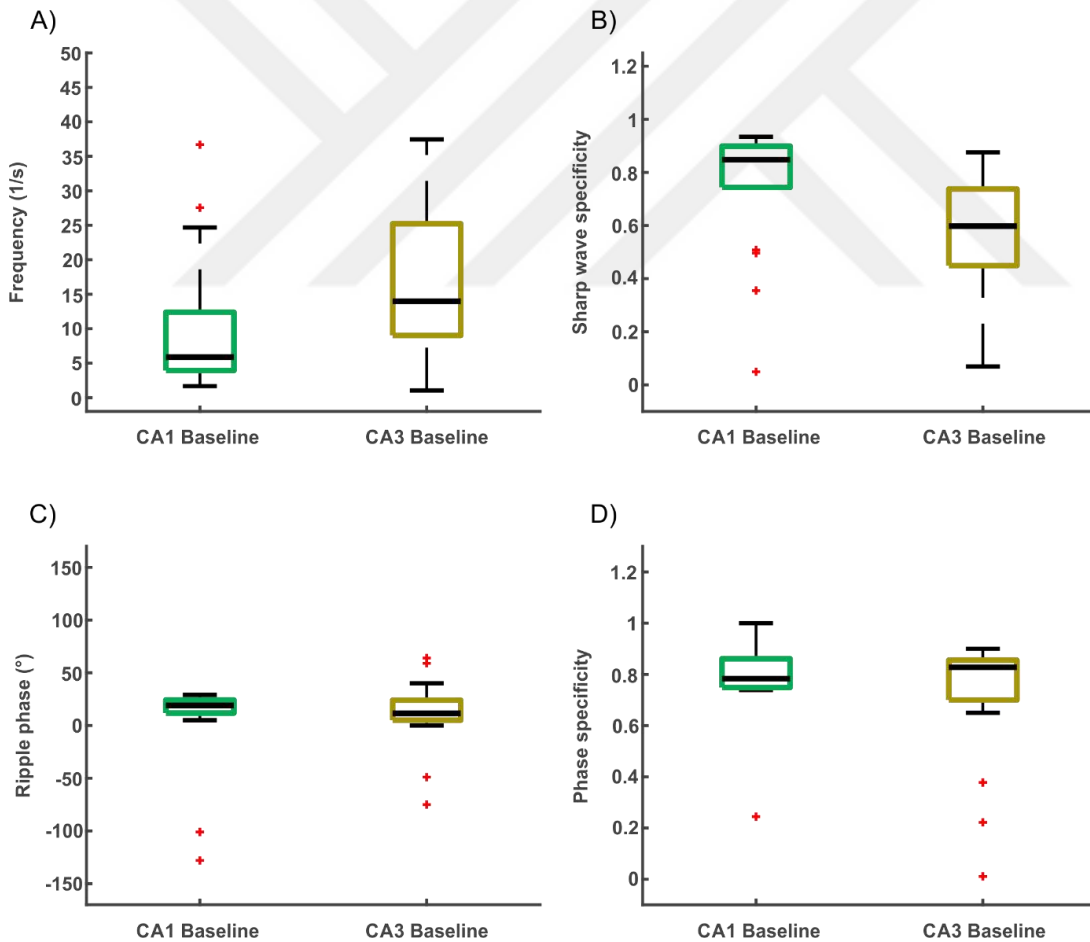


Figure 12: Baseline properties of MUA in CA1 and CA3. A-D) MUA property boxplots, in all boxplots, the MUA properties shown from left to right are; CA1 Baseline (light green colored), CA3 Baseline (light brown colored). **A)** Frequency. **B)** Sharp wave specificity. **C)** Ripple phase. **D)** Ripple phase specificity. $n_{CA1} = 20$ slices from 11 mice, $n_{CA3} = 24$ slices from the same 11 mice.

MUA frequency of the CA1 region during baseline was 5.88/s (Hz) [3.93 12.39] and MUA frequency of the CA3 region during baseline was 13.98/s (Hz) [9.02 25.22] (Figure 12, A). MUA sharp wave specificity of the CA1 region during baseline was 0.85 [0.74 0.90] and MUA sharp wave specificity of the CA3 region during baseline was 0.60 [0.45 0.74] (Figure 12, B). MUA median ripple phase of the CA1 region during baseline was 19 degrees (°) [11.75 24.25], and MUA median ripple phase of the CA3 region during baseline was 11.5 degrees (°) [5 24] (Figure 12, C). MUA ripple phase specificity of the CA1 region during baseline was 0.78 [0.75 0.86] and MUA ripple phase specificity of the CA3 region during baseline was 0.83 [0.70, 0.86] (Figure 12, D).

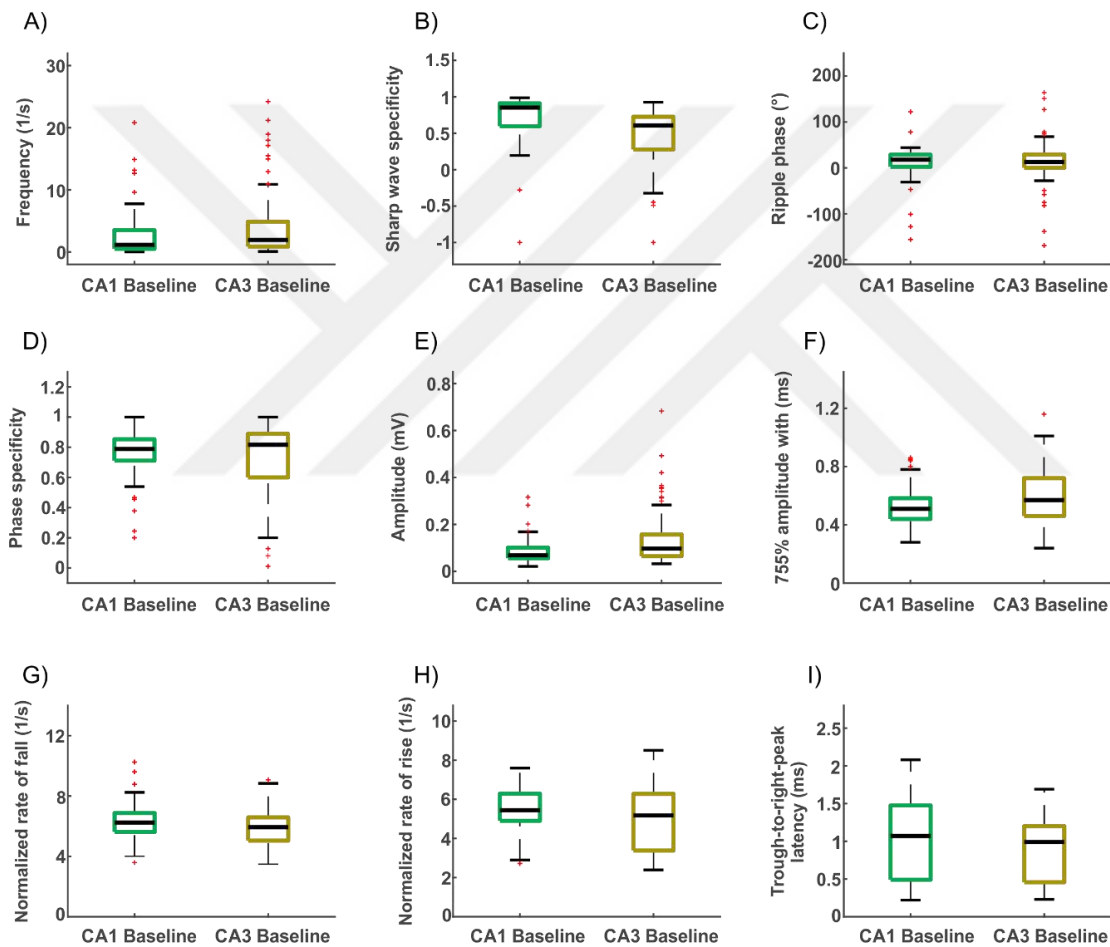


Figure 13: Baseline properties of single units in CA1 and CA3. A-I) Unit property boxplots, in all boxplots the unit properties shown from left to right are; CA1 Baseline (light green colored), CA3 Baseline (light brown colored). **A)** Frequency. **B)** Sharp wave specificity. **C)** Ripple phase. **D)** Ripple phase specificity. **E)** Amplitude **F)** Width75. **G)** Normalized rate of fall. **H)** Normalized rate of rise. **I)** Trough-to-right-peak latency. $n_{CA1} = 77$ units taken from 21 slices from 11 mice. $n_{CA3} = 105$ units from 24 slices from the same 11 mice.

Frequency of the CA1 units during baseline was 1.15/s (Hz) [0.50 3.51] and frequency of the CA3 units during baseline was 1.93/s (Hz) [0.86 4.88] (Figure 13, A). Sharp wave specificity of the CA1 units during baseline was 0.85 [0.60 0.91] and sharp wave specificity of the CA3 units during baseline was 0.61 [0.28 0.73] (Figure 13, B). Median ripple phase of the CA1 units during baseline was 18 degrees (°) [2.25 28.75] and median ripple phase of the CA3 units during baseline was 13 degrees (°) [0 29] (Figure 13, C). Ripple phase specificity of the CA1 units during baseline was 0.79 [0.71 0.85] and ripple phase specificity of the CA3 units during baseline was 0.82 [0.60 0.89] (Figure 13, D).

Amplitude of the CA1 units during baseline was 0.069 mV [0.056 0.10] and amplitude of the CA3 unit during baseline was 0.097 mV [0.065 0.16] (Figure 13, E). 75 % amplitude width (width 75) of the CA1 units during baseline was 0.51 milliseconds (ms) [0.44 0.58] and 75 % amplitude width (width 75) of the CA3 units during baseline was 0.57 ms [0.46 0.72] (Figure 13, F). Normalized rate of fall (fall-norm) of the CA1 units during baseline was 6.24 /s [5.62 6.87] and normalized rate of fall (fall-norm) of the CA3 units during baseline was 5.93/s [5.04 6.58] (Figure 13, G). Normalized rate of rise (rise-norm) of the CA1 units during baseline was 5.45/s [4.90 6.29] and normalized rate of rise (rise-norm) of the CA3 units during baseline was 5.18/s [3.38 6.28] (Figure 13, H). Trough-to-right-peak latency (tRightPeak) of the CA1 units during baseline was 1.07 ms [0.49 1.48] and trough-to-right-peak latency (tRightPeak) of the CA3 units during baseline was 0.99 ms [0.46 1.20] (Figure 13, I).

After baseline 20 minute recordings were taken, mild OGD was performed on the experiment slices and the latency from the start of OGD to the Spreading Depression (SD) was recorded. The median latency time from OD start to SD, from the 15 slices that underwent OGD, was 405 [358.25, 448.25] seconds, which is in line with the pervious experiments that used young adult mice (Grube et.al, manuscript in print) (Figure 14).

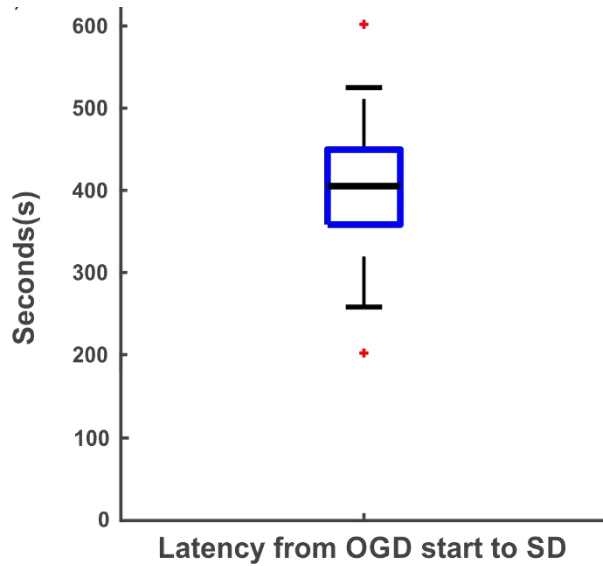


Figure 14: Latency from OGD start to SD. Latency from the start of OGD to Spreading Depression (SD) boxplot. Median= 405 seconds [358.25, 448.25]. n = 15 slices from 11 mice aged 75 weeks to 104 weeks.

3.1.1.1. SPW-R frequency and unit firing is reduced in the OGD slices one hour post SD

After the OGD slices were put through mild OGD until spreading depression, the one hour recovery of the CA1 and CA3 regions were recorded continuously and the last 20 minutes of the 60 minutes after the spreading depression was analyzed using custom algorithms on MATLAB for the change of the network oscillation properties of these slices (figure 15). Because the recordings were made continuously there are lines connecting the values of the boxplots on Figure 15, to show that baseline and one hour after spreading depression (OGD 1 h) values are matched pairs and also to show the change of each slice more clearly. These lines connecting baseline values to their corresponding OGD 1h values can also be seen in the MUA (Figure 16) and unit activity (Figure 17) boxplots as same principle can be applied

Figure 15 shows that no property of network oscillation other than sharp wave frequency changed significantly ($p > 0.05$). For both CA1 and CA3 regions, there was a significant reduction of sharp wave frequency one hour after the spreading depression (Figure 15, E) ($p_{CA1} = 0.020$, $p_{CA3} = 0.016$). This is in line with previous findings showing a disruption of SPW-R after mild OGD (Grube et.al, manuscript in print). There was no significant change in ripple frequency (Figure 15, B), ripple energy (Figure 15, C), sharp wave amplitude (Figure 15, D) and sharp wave width (Figure 15, F) from baseline to OGD 1h for both CA1 and CA3 regions.

From the analysis of the continuous recording of the OGD slices from start of OGD to one hour post SD, a binned median frequency over time plot can be made which show that after the start of the OGD, there is a sharp decrease in sharp wave frequency, (around the time of the Spreading Depression), and that there is a gradual increase in frequency of both CA1 and CA3 regions along the one hour recovery. However, the frequency doesn't return to the frequency prior to start of OGD at one hour post SD. (Figure 15, G, and H). These frequency over time plots were normalized to the sharp wave frequency of the baseline 20 minutes so 1 represents the value of the baseline sharp wave frequency.

MUA frequency, ripple phase and phase specificity of CA1 change significantly at one hour post SD.

MUA frequency of the CA1 region was reduced significantly ($p = 0.012$) from baseline to OGD 1h and for the CA3 region there was also an almost significant decrease of MUA frequency ($p = 0.064$), (Figure 16, A). This shows that at one hour post SD, not only is there a disruption of the SPW-R but a disruption of individual cell firing as well. So there seems to be a suppression of both network and single cell activity following the initial one hour recover from SD.

For the CA1 region there was a significant negative change in the median ripple phase where multi-unit activity (MUA) occurred from baseline to one hour post SD ($p = 0.031$) (Figure 16, C). There was also a significant decrease in ripple phase specificity for the CA1 region (Figure 16, D) ($p = 0.031$) (Figure 16, D). This indicates that there was a disruption in the coupling of MUA to the ripple phase in CA1 but not in the CA3 region. There was no significant change in sharp wave specificity of MUA from baseline to one hour post SD for both CA1 and CA3 regions (Figure 16, B).

The decrease in MUA frequency can also be seen by looking at the binned MUA frequency over time plots again deduced from the continuous tetrode recordings from start of OGD to one hour post SD (Figure 16, E, and F). Looking at these plots, a sharp increase in MUA frequency prior to SD can be seen, which is caused by the spike bursting just prior to SD. This is followed by a sharp decrease in MUA frequency as it was for sharp wave frequency, which is followed by a gradual increase over time. These frequency over time plots were normalized to the MUA frequency of the baseline 20 minutes so 1 represents the value of the baseline MUA frequency. For CA1, after a gradual increase the final MUA frequency at one hour post SD is less than the frequency at baseline (Figure 16, E). For CA3 the MUA frequency rises sharply to baseline

values after SD and gradually declines to a little bit less than the baseline frequency at one hour post SD with more oscillation of the frequency than the CA1 region (Figure 16, F).

Unit frequency is significantly reduced in both CA1 and CA3 regions and CA1 unit to SPW-R coupling is significantly increased at one hour post SD.

At one hour post SD, it was observed that the unit frequency in both CA1 and CA3 was significantly reduced ($p_{CA1} = 0.0013$, $p_{CA3} = 0.0068$.) (Figure 17, A). This result aligns with the MUA frequency decrease of the slices at one hour post SD, which was significant for CA1 and almost significant for the CA3 region. This decrease in unit frequency at one hour post SD clearly shows that mild OGD causes disruption to both network oscillations and unit firing and it is known that SPW-R oscillation is closely connected to unit firing so this result is not surprising (Both et al., 2008, Buzsáki, 2015). Looking closely at the lines connecting each value, it can be seen that the reduction of frequency at one hour post SD for the units with higher baseline frequency is significant and the units with lower baseline frequency seems to have stayed the same (Figure 17, A). This indicates that the reduction of frequency at one hour post SD seems to happen more strongly for fast-spiking units (interneurons) rather than for the slow-spiking units (pyramidal cells), which is in line with previous findings (Grube et al., manuscript in print). Median ripple phase degree where units fired also decreased significantly for the CA1 region ($p = 0.019$) (Figure 17, C) which aligns with the significant decrease of the median ripple phase of MUA for the CA1 region (Figure 16, C). Unlike MUA, there wasn't any significant reduction of ripple phase specificity of unit firing for the CA1 region (Figure 17, D).

Sharp wave specificity (SPW-R coupling) of the CA1 units was also significantly increased at one hour post SD ($p = 0.0067$) (Figure 17, B). This coincides with the reduction of frequency of the CA1 units, suggesting that units were mostly able to fire were only during sharp waves. This increase in sharp wave specificity was not observed for the CA3 units.

There was a significant increase in the 75 % amplitude width (Width75) of the units of the CA3 region ($p = 0.048$) at one hour post SD although there was no such significant change for the units of the CA1 region from baseline to one hour post SD (Figure 17, F). There was also a significant increase in the normalized rate of fall (fall-norm) of the units of the CA1 region ($p = 5.10 \times 10^{-4}$) although there was no such significant change for the units of the CA3 region (Figure 17, G). There was no significant change in the phase specificity (Figure 17, D), amplitude (Figure 17, E), normalized rate of the rise (rise-norm) (Figure 17, G) and trough-to-

right-peak latency (tRightPeak) (Figure 17, I) from baseline to one hour post SD for both CA1 and CA3 regions.

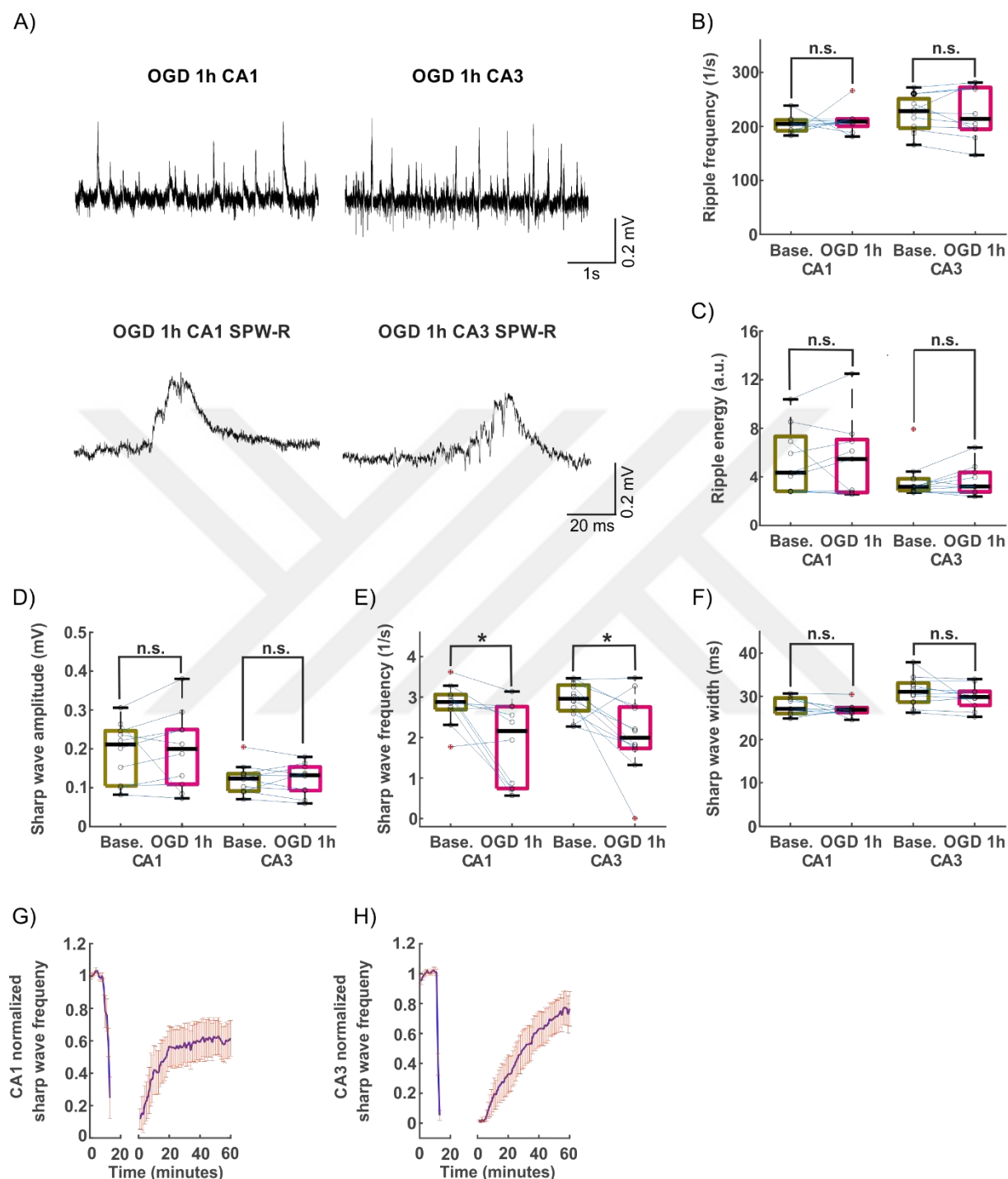


Figure 15: Example traces of tetraode recordings taken from CA1 and CA3 stratum pyramidale of OGD slices at one hours post SD and properties of SPW-R in CA1 and CA3, during baseline and one hour post SD. A) Top: Example traces of 5 second tetraode recordings taken from CA1 (left) and CA3 (right) at Baseline. Bottom: Example traces of 100 milliseconds tetraode recordings showing a single SPW-R taken from CA1 (left) and CA3 (right) at OGD 1 h (one hour post SD). **B-F)** Network oscillation property boxplots comparing Baseline and OGD 1hour (one hour post SD) for both CA1 and CA3 regions. In all boxplots network oscillation properties shown from left to right in each plot are;

CA1 Baseline (light green colored), CA1 OGD 1hour (pink colored), CA3 Baseline (light green colored) and CA3 OGD 1hour (pink colored). All p values are calculated using the Wilcoxon signed-rank test. **B)** Ripple frequency, $p_{CA1} = 0.64$, $p_{CA3} = 0.70$. **C)** Ripple energy, $p_{CA1} = 0.73$, $p_{CA3} = 0.23$. **D)** Sharp wave amplitude, $p_{CA1} = 0.23$, $p_{CA3} = 0.92$. **E)** Sharp wave frequency, $p_{CA1} = 0.020$, $p_{CA3} = 0.016$. **F)** Sharp wave width, $p_{CA1} = 0.41$, $p_{CA3} = 0.10$. **G)** Binned mean CA1 Sharp wave Frequency normalized to baseline frequency. Shaded region represents standard error of the mean. 0-20= 20 minutes from Spreading Depression (SD). Gap= SD, signifying the variability of the duration of SD. 0-60 = 60 minutes immediately after SD event and reperfusion with ACSF and Oxygen. **H)** Binned mean CA3 Sharp wave Frequency normalized to baseline frequency. Shaded region represents standard error of the mean. 0-20= 20 minutes from Spreading Depression (SD). Gap= SD, signifying the variability of the duration of SD. 0-60 = 60 minutes immediately after SD event and reperfusion with ACSF and Oxygen. $n_{CA1} = 10$ slices from 11 mice, $n_{CA3} = 12$ slices from the same 11 mice.

Base. = Baseline, n.s. = not significant, * = $p < 0.05$, $p = p$ value, a.u. =arbitrary unit

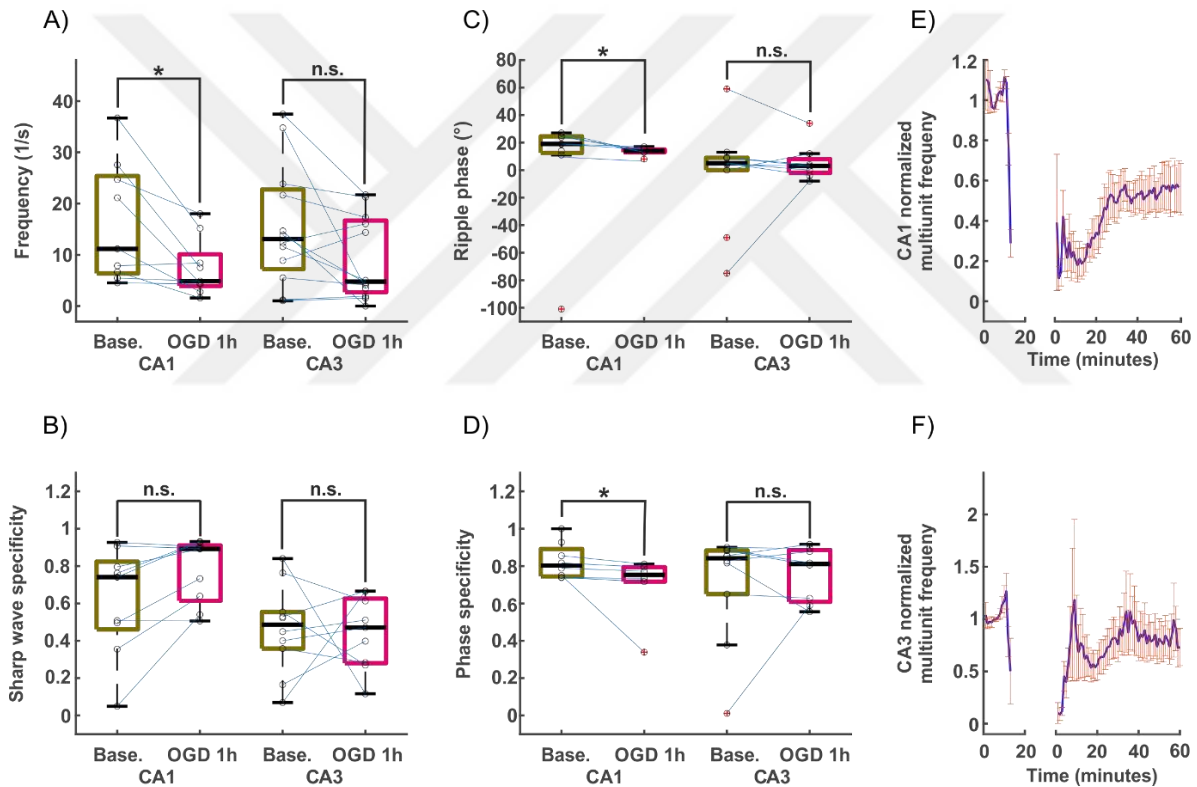


Figure 16: Properties of MUA in CA1 and CA3 during baseline and one hour post SD. A-D) MUA property boxplots, comparing Baseline and OGD 1hour for both CA1 and CA3 regions. In all boxplots the MUA shown from left to right in each plot are; CA1 Baseline (light green colored), CA1 OGD 1hour (pink colored), CA3 Baseline (light green colored) and CA3 OGD 1hour (pink colored). All p values are calculated using the Wilcoxon signed-rank test. **A)** Frequency, $p_{CA1} = 0.012$, $p_{CA3} = 0.064$. **B)** Sharp wave specificity, $p_{CA1} = 0.055$, $p_{CA3} = 0.91$. **C)** Ripple phase, $p_{CA1} = 0.031$, $p_{CA3} = 0.61$. **D)** Ripple phase specificity, $p_{CA1} = 0.031$, $p_{CA3} = 0.74$. **E)** Binned mean CA1 MUA Frequency normalized to baseline frequency. Shaded region represents standard error of the mean. 0-20= 20 minutes from Spreading Depression (SD). Gap= SD, signifying the variability of the duration of SD. 0-60 = 60 minutes immediately after SD event and reperfusion with ACSF and Oxygen. **F)** Binned mean CA3 MUA Frequency normalized to baseline frequency. Shaded region represents standard error of the

mean. 0-20= 20 minutes from Spreading Depression (SD). Gap= SD, signifying the variability of the duration of SD. 0-60 = 60 minutes immediately after SD event and reperfusion with ACSF and Oxygen. n_{CA1} = 9 slices from 11 mice, n_{CA3} = 12 slices from the same 11 mice.

Base. = Baseline, n.s. = not significant, * = $p < 0.05$, p = p value

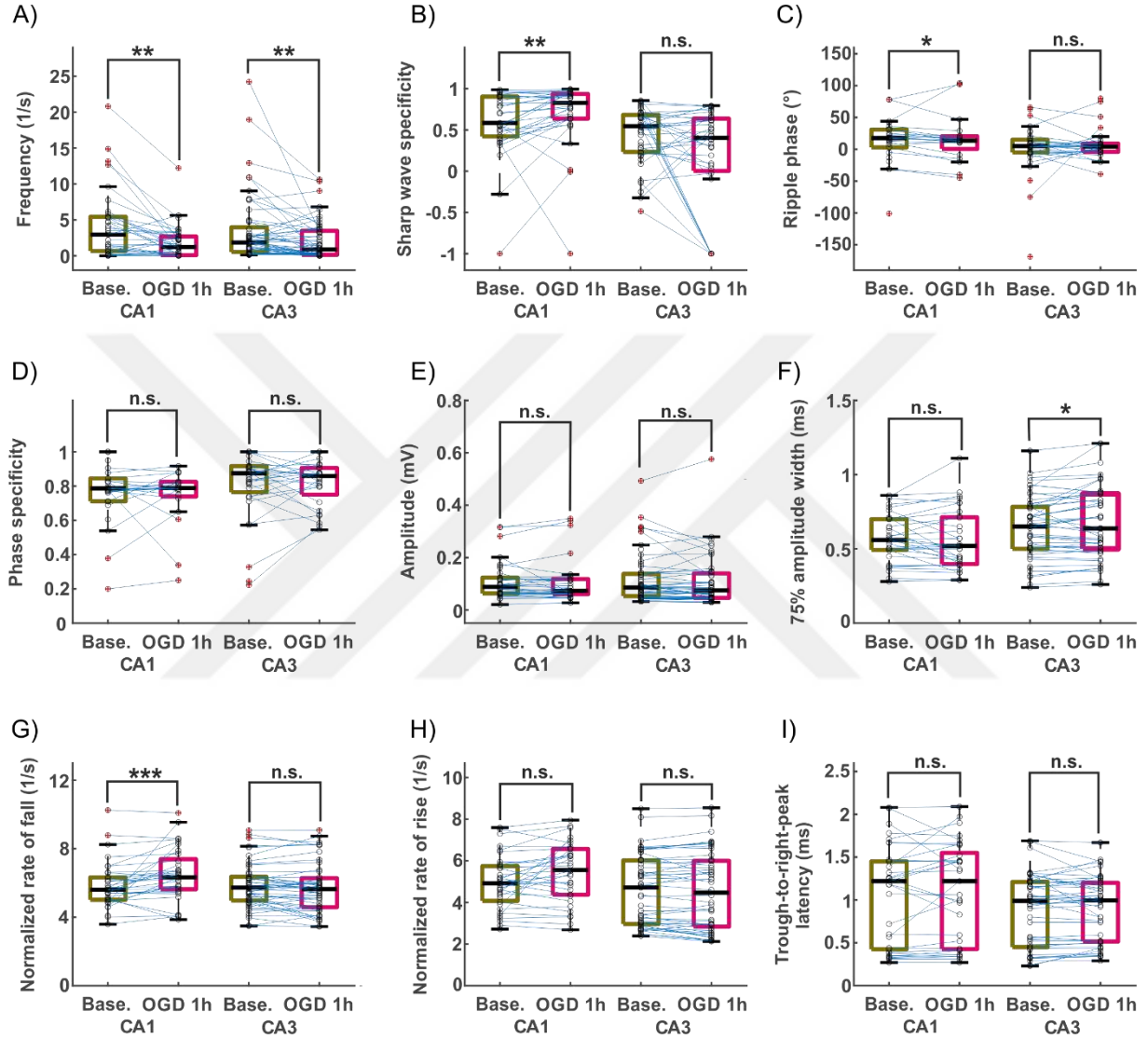


Figure 17: Properties of single units in CA1 and CA3 during baseline and one hour post SD. A-I) Boxplots of Unit properties comparing Baseline and OGD 1hour for both CA1 and CA3 regions. In all boxplots units shown from left to right are; CA1 Baseline (light green colored), CA1 OGD 1hour (pink colored), CA3 Baseline (light green colored) and CA3 OGD 1hour (pink colored). All p values are calculated using the Wilcoxon signed-rank test. **A)** Frequency, $p_{CA1} = 0.0013$, $p_{CA3} = 0.0068$. **B)** Sharp wave specificity, $p_{CA1} = 0.0067$, $p_{CA3} = 0.19$. **C)** Ripple phase, $p_{CA1} = 0.019$, $p_{CA3} = 0.59$. **D)** Ripple phase specificity, $p_{CA1} = 0.40$, $p_{CA3} = 0.71$. **E)** Amplitude, $p_{CA1} = 0.99$, $p_{CA3} = 0.22$. **F)** 75 % amplitude width, $p_{CA1} = 0.58$, $p_{CA3} = 0.048$. **G)** Normalized rate of fall, $p_{CA1} = 5.10 \times 10^{-4}$, $p_{CA3} = 0.24$. **H)** Normalized rate of rise, $p_{CA1} = 0.059$, $p_{CA3} = 0.40$. **I)** Trough-to-right-peak latency, $p_{CA1} = 0.40$, $p_{CA3} = 0.67$. $n_{CA1} = 35$ units taken from 9 slices from 11 mice. $n_{CA3} = 53$ units from 12 slices from the same 11 mice.

Base. = Baseline, n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, p = p value

3.1.1.2. SPW-R frequency of OGD slices were lower compared to control slices at four hours post SD, whereas unit firing of OGD slices were higher

After mild OGD was performed and one hour recovery of OGD slices were recorded, the OGD and control slices were left to recover further in their respective Haas-type interface chambers. Then, another 20 minute tetrode recording was made from 3 hours 40 minutes after the onset of SD to four hours after the onset of SD. These recordings were taken from both OGD and control slices and from both the CA1 and CA3 pyramidal cell layers (stratum pyramidale). To make sense of the change of the network oscillation and multi-unit activity (MUA) at four hours post SD, the values were normalized to baseline by dividing the OGD 4h and control 4h values of network oscillation properties to the baseline values of the same slice. Therefore, for the boxplots shown in Figure 18, a value of 1 indicates the baseline value.

The normalized sharp wave frequency of the CA3 region of OGD 4h slices was significantly lower compared to the normalized sharp wave frequency of the control 4h slices ($p = 0.0035$) (Figure 18, E). For the CA1 region the OGD 4h slices did have lower normalized sharp wave frequency as well and it was close to being a significant difference ($p = 0.073$) (Figure 18, E) that might have been significant had there been more slices. This finding indicates that the disruption to the network oscillation cause by the mild OGD is still effecting the slices after four hour recovery and seems to have a lasting effect.

There was no significant difference in normalized ripple frequency (Figure 18, B), normalized ripple energy (Figure 18, C) and normalized sharp wave amplitude (Figure 18, D) between OGD 4h and control 4h slices for both CA1 and CA3 regions. Sharp wave width of the CA3 region of the control 4h slices was significantly larger compared to OGD 4h slices ($p = 0.044$) and sharp wave width of the CA1 region of the control 4h slices was almost significantly larger compared to OGD 4h slices ($p = 0.065$) (Figure 18, F).

The change of MUA Ripple Phase was significant in the CA3 region for OGD 4h slices compared to control 4h slices

To make sense of the change in the MUA ripple activity properties, the values for OGD 4h and control 4h were normalized to baseline values as was done for network oscillation values. For the MUA frequency this was done by dividing the MUA frequency of OGD and control 4h slices were to the baseline value of the same slice so a value of 1 indicates the baseline value (Figure 19, A). For the ripple phase, ripple phase specificity and sharp wave specificity, the change was calculated by subtracting the baseline value of the same slice from the OGD and

control 4h value. So for the change of ripple phase, sharp wave specificity and ripple phase specificity, a value of 0 indicates that OGD 4h value is the same as the baseline value (Figure 19, B, C, and D).

The change of the median ripple phase of MUA was positive for the CA3 region of the OGD 4h slices and negative for the control 4h slices and this difference in the change of median ripple phase was significant between OGD 4h and control 4h slices ($p= 0.033$) (Figure 19, C). This positive change of the MUA median ripple phase of the CA3 region is in the opposite direction of MUA median ripple phase change of the CA1 region of OGD 1h, where there was a negative change in the degree of the median ripple phase from baseline to one hour post SD (Figure 16, C). The change of median ripple phase degree is negative for the CA1 region of OGD 4h slices so the decrease of the median ripple phase degree compared to baseline stayed consistent for the CA1 region (Figure 19, C). There was no significant difference in normalized frequency (Figure 19, A), change of sharp wave specificity (Figure 19, B) and change of ripple phase specificity (Figure 19, D) between MUA of OGD 4h and control 4h slices for both CA1 and CA3 regions.

Unit frequency of the CA3 region was significantly higher for the OGD 4h slices compared to control 4h slices and unit to SPW-R coupling of the CA1 region was significantly smaller for the OGD 4h slices compared to control 4h slices.

Since at four hours post SD, a new 20 minute tetrode recording was made, the units deduced from these recordings are not the same units as were recorded from baseline to one hour post SD. Furthermore, unlike network oscillation and MUA properties which are general properties of the slice, the unit activity properties of the OGD and control 4h units can't be normalized to their baseline values since they are completely different units than the units recorded during baseline. So the unit activity properties of the OGD and control 4h of the CA1 and CA3 regions were compared to each other without normalizing to baseline.

Unit frequency of the CA3 region of OGD 4h slices were significantly higher than the unit frequency of control 4h slices ($p= 0.016$) (Figure 20, A). The CA1 region unit frequency was also higher for OGD 4h slices even though the change was not significant ($p=0.15$) (figure 20, A). This finding was unexpected and is different than previous findings of the effect of mild OGD on unit activity (Grube et.al, manuscript in print). However, it must be noted that the previous experiment (Grube et al., manuscript in print) only had one hour recovery to baseline comparisons and the slices were not left to recover until four hours post SD.

Sharp wave specificity (unit to SPW-R coupling) of the CA1 units of the OGD 4h slices were significantly smaller than the CA1 units of the control 4h slices ($p=0.014$) (Figure 20, B). This finding is the opposite of the one hour post SD values which showed an increase in sharp wave specificity compared to baseline at one hour post SD. At one hour post SD, unit frequency was reduced compared to baseline for both CA1 and CA3 regions but at four hours post SD, CA1 unit frequency is larger for the OGD 4h slices compared to control 4h slices. This suggests that as the units tend to fire more frequently, the firing tends to become less coupled to SPW-R. This reduction of sharp wave specificity compared to control 4h also indicates that unit coupling to network oscillation is disrupted even four hours after the onset of SD, which might have a detrimental effect to the information coding of the hippocampal network.

The amplitude of units of the CA3 region of control 4h slices were significantly larger than the units of OGD 4h slices ($p=2.70 \times 10^{-4}$) (Figure 20, E). There was no significant difference in the median ripple phase (Figure 20, C), ripple phase specificity (Figure 20, D), 75 % amplitude width (width75) (Figure 20, F), normalized rate of fall (fall-norm) (Figure 20, G), normalized rate of rise (rise-norm) (Figure 20, H) and trough-to-right-peak latency (Figure 20, I) between OGD 4h and control 4h slices for the units of CA1 and CA3 regions.

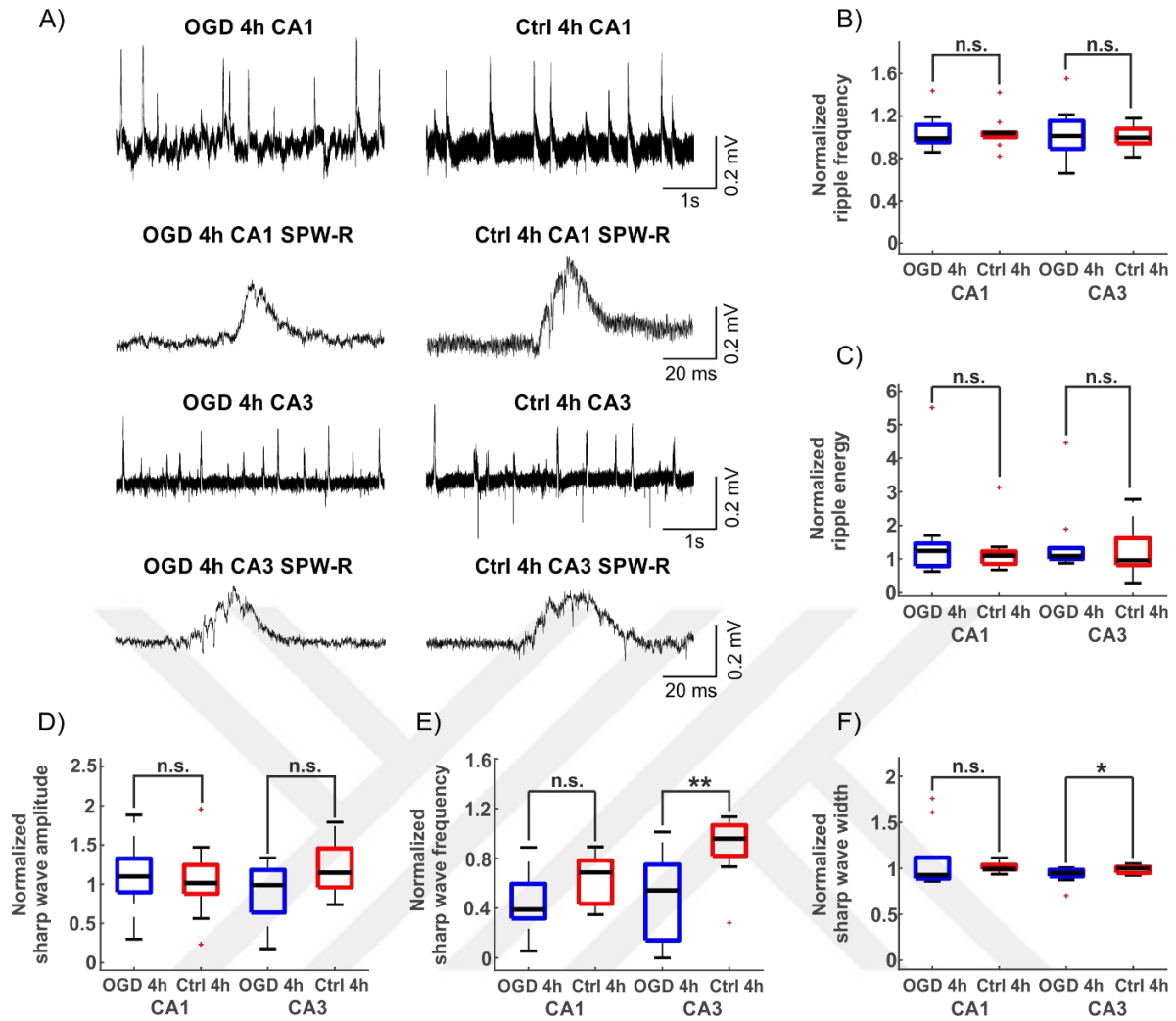


Figure 18: Example traces of tetrode recordings taken from CA1 and CA3 stratum pyramidale of OGD and control slices at four hours post SD and properties of SPW-R in CA1 and CA3, comparing OGD and control slices four hours post SD. **A)** Top: Example traces of 5 second tetrode recordings taken from OGD 4h (four hours post SD) CA1 (left) and control 4h CA1 (right). 2nd row: Example traces of 100 milliseconds tetrode recordings showing a single Sharp wave-Ripple Complex (SPW-R) taken from OGD 4h CA1 (left) and control 4h CA1 (right). 3rd row: Example traces of 5 second tetrode recordings taken from OGD 4h (four hours post OGD) CA3 (left) and control 4h CA3 (right). 4th row: Example traces of 100 milliseconds tetrode recordings showing a single sharp wave-ripple complex (SPW-R) taken from OGD 4h CA3 (left) and control 4h CA3 (right). **B-F)** Normalized network oscillation property boxplots comparing OGD 4hour (four hours post SD) and control 4h for both CA1 and CA3 regions. In all boxplots, network oscillation properties shown from left to right in each plot are; CA1 OGD 4hour (blue colored), CA1 control 4h (red colored), CA3 OGD 4hour (blue colored), CA3 control 4hour (red colored). Values are normalized to baseline by dividing the OGD and control 4hour values by the baseline values of their respective slices. All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. **B)** Ripple frequency, $p_{CA1} = 0.59$, $p_{CA3} = 0.77$. **C)** Ripple energy, $p_{CA1} = 0.66$, $p_{CA3} = 0.28$. **D)** Sharp wave amplitude, $p_{CA1} = 0.60$, $p_{CA3} = 0.069$. **E)** Sharp wave frequency, $p_{CA1} = 0.073$, $p_{CA3} = 0.0035$. **F)** Sharp wave width, $p_{CA1} = 0.065$, $p_{CA3} = 0.044$. $n_{\text{OGD4hCA1}} = 10$ slices from 11 mice. $n_{\text{OGD4hCA3}} = 12$ slices from the same 11 mice. $n_{\text{CTRL4hCA1}} = 11$ slices from 11 mice. $n_{\text{CTRL4hCA3}} = 12$ slices from the same 11 mice.

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, p = p value, Ctrl = control

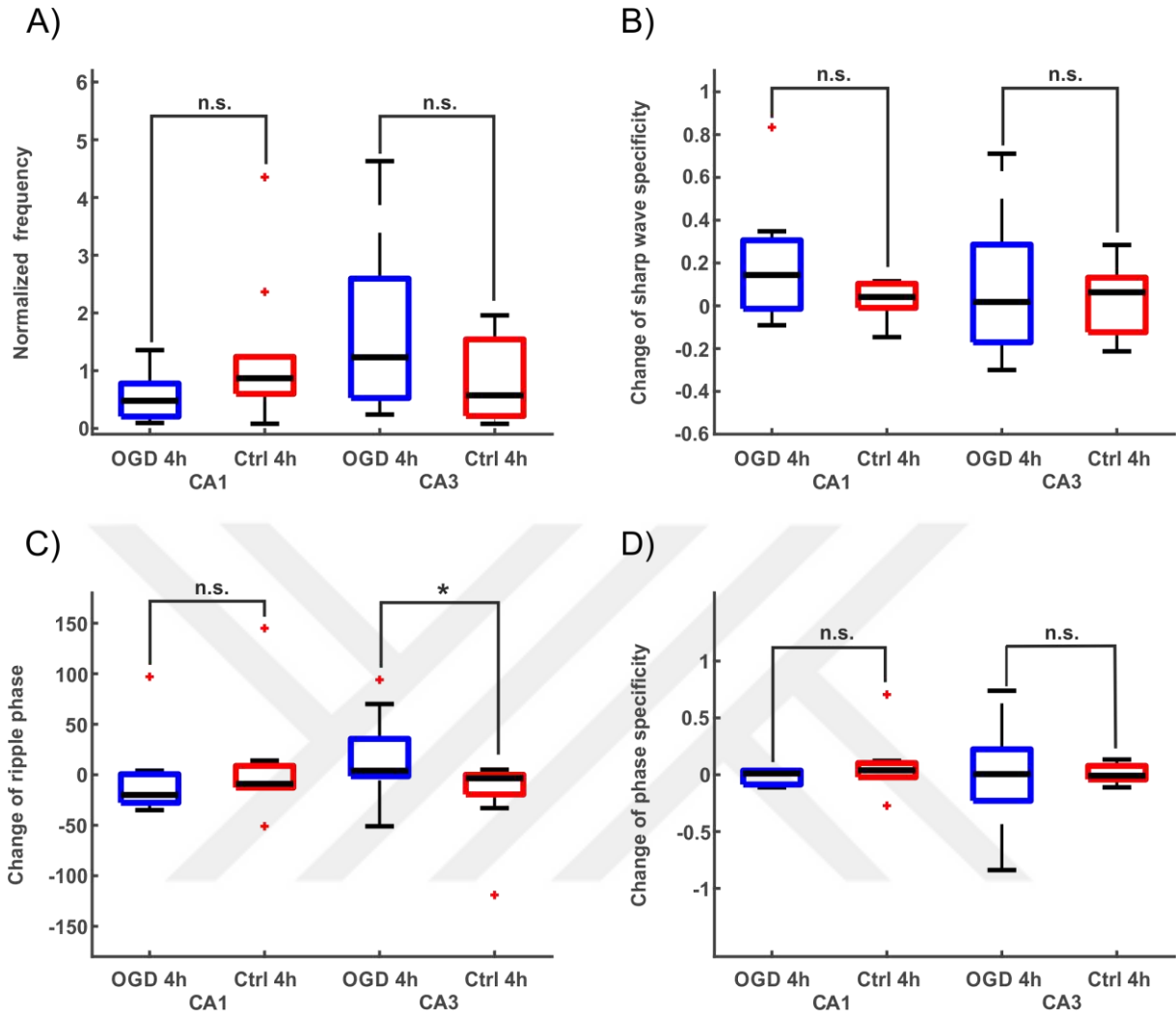


Figure 19: Properties of MUA in CA1 and CA3, comparing OGD and control slices four hours post SD. A-D) Normalized MUA activity properties, comparing OGD 4hour and control 4hour for both CA1 and CA3 regions. In all boxplots, the MUA shown from left to right are CA1 OGD 4hour (blue colored), CA1 control 4hour (red colored), CA3 OGD 4hour (blue colored) and CA3 control 4hour (red colored). Values are normalized to baseline. All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test unless specified otherwise. **A)** Normalized frequency, calculated as the OGD and control 4hour values divided by baseline values of their respective slices, $p_{CA1} = 0.18$, $p_{CA3} = 0.06$. **B)** Change of sharp wave specificity, calculated as the OGD and control 4hour values subtracted from the baseline values of their respective slices, $p_{CA1} = 0.15$ (unpaired Student's t-test), $p_{CA3} = 0.92$ (Wilcoxon rank-sum) test **C)** Change of median ripple phase, calculated as the OGD and control 4hour values subtracted from the baseline values of their respective slices, $p_{CA1} = 0.46$, $p_{CA3} = 0.033$. **D)** Change of ripple phase specificity, calculated as the OGD and control 4hour values subtracted from the baseline values of their respective slices, $p_{CA1} = 0.37$ (unpaired Student's t-test), $p_{CA3} = 0.97$ (Wilcoxon rank-sum test). $n_{OGD4hCA1} = 9$ slices from 11 mice. $n_{OGD4hCA3} = 10$ slices from the same 11 mice. $n_{CTRL4hCA1} = 10$ slices from 11 mice. $n_{CTRL4hCA3} = 12$ slices from the same 11 mice.

n.s. = not significant, * = $p < 0.05$, p = p value Ctrl = control

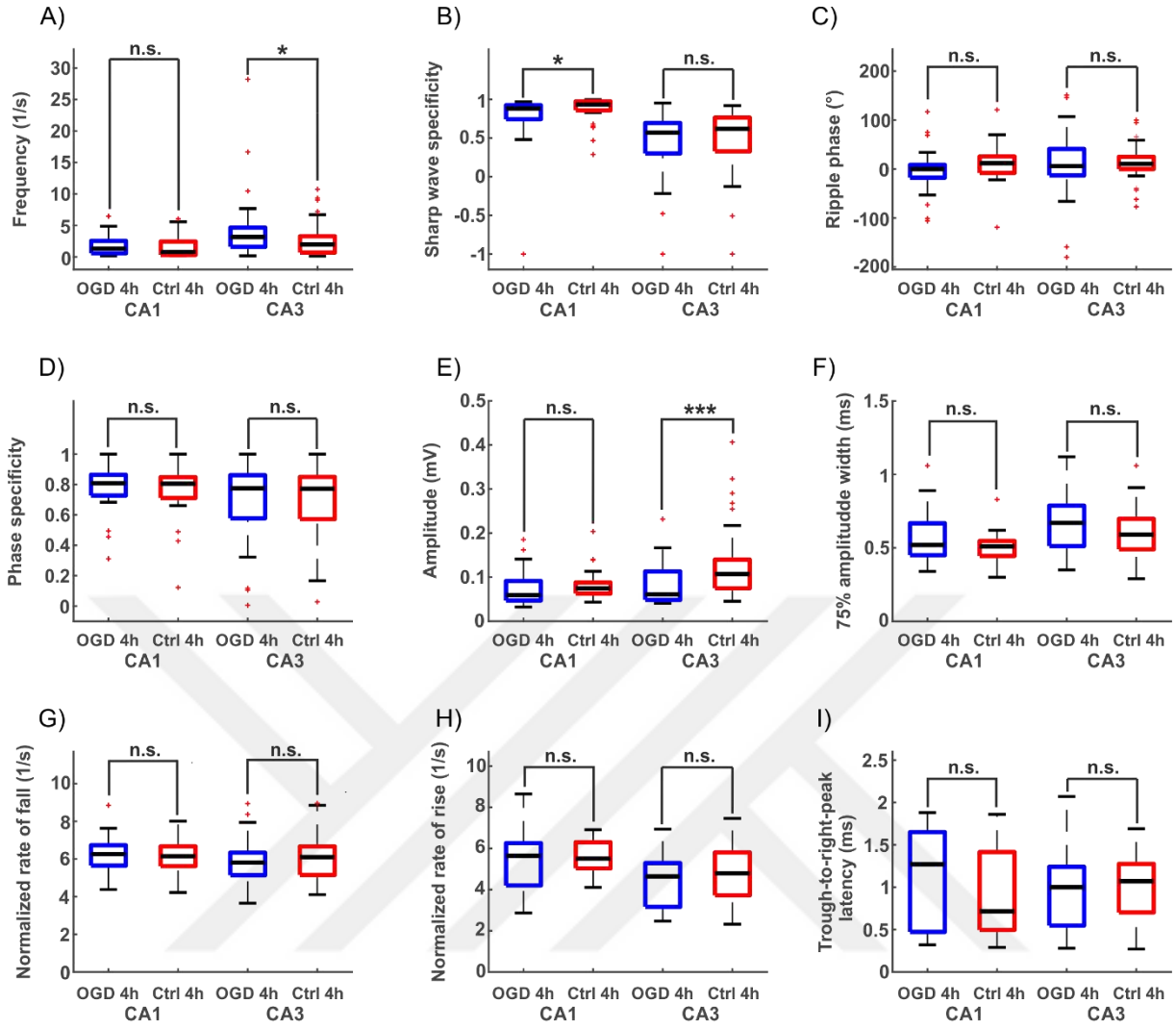


Figure 20: Properties of single units in CA1 and CA3, comparing OGD and control slices four hours post SD. A-I) Unit property boxplots comparing OGD 4hour and control 4hour for both CA1 and CA3 regions. In all boxplots units shown from left to right are; CA1 OGD 4hour (blue colored), CA1 control 4hour (red colored), CA3 OGD 4hour (blue colored) and CA3 control 4hour (red colored). All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. **A)** Frequency, $p_{CA1} = 0.15$, $p_{CA3} = 0.016$. **B)** Sharp wave specificity, calculated as $(f_{\text{inside}} - f_{\text{outside}}) / (f_{\text{inside}} + f_{\text{outside}})$, $p_{CA1} = 0.014$, $p_{CA3} = 0.41$. **C)** Ripple phase, $p_{CA1} = 0.079$, $p_{CA3} = 0.46$. **D)** Ripple phase Specificity, $p_{CA1} = 0.61$, $p_{CA3} = 0.99$. **E)** Amplitude, $p_{CA1} = 0.074$, $p_{CA3} = 2.70 \times 10^{-4}$. **F)** 75 % amplitude width, $p_{CA1} = 0.16$, $p_{CA3} = 0.094$. **G)** Normalized rate of fall, $p_{CA1} = 0.98$, $p_{CA3} = 0.17$. **H)** Normalized rate of rise, $p_{CA1} = 0.44$, $p_{CA3} = 0.12$. **I)** Trough-to-right-peak latency, $p_{CA1} = 0.27$, $p_{CA3} = 0.62$. $n_{\text{OGD4hCA1}} = 39$ units taken from 10 slices from 11 mice, $n_{\text{CTRL4hCA1}} = 32$ units taken from 10 slices from 11 mice, $n_{\text{OGD4hCA3}} = 51$ units from 10 slices from the same 11 mice, $n_{\text{CTRL4hCA3}} = 55$ units from 12 slices from the same 11 mice.

n.s. = not significant, * = $p < 0.05$, *** = $p < 0.001$, p = p value, Ctrl = control

3.1.2. Mild OGD leads to an increase in PYR firing and a decrease in FSI firing

Baseline properties of PYR, FSI and MIX units

As discussed in the Methods section, the units analyzed by the Wave_clus algorithm were further separated into 3 classes to better analyze the effects of mild OGD on different neuron types of the hippocampus. The slow-spiking pyramidal cells (PYR), the fast-spiking interneurons (FSI) and units that could not be separated into PYR or FSI classes which contain a mixture of pyramidal cells and interneurons (MIX). The three unit firing properties that were used to separate units were burst percentage, log of ISI, and trough-to-right-peak latency (tRightPeak) (for further explanation, see Method). FSI are expected to have a smaller log of ISI and a larger Burst Percentage than PYR which can be seen for slices during baseline conditions (Sirota et al., 2008; English et al., 2017) (Figure 21, B,C). The FSI have the smallest log of ISI and the largest burst percentage as can be seen from Figure 21, the PYR have the largest log of ISI and the smallest burst percentage and the MIX cells have values somewhere in between PYR and FSI, indicating that the segregation of units worked as expected. As explained in the methods section, FSI are expected to have the smallest trough-to-right-peak latency (tRightPeak) values (Sirota et al., 2008; English et al., 2017) and during baseline conditions this is observed (Figure 21, I). This is another indication that separation of units into different classes worked as expected.

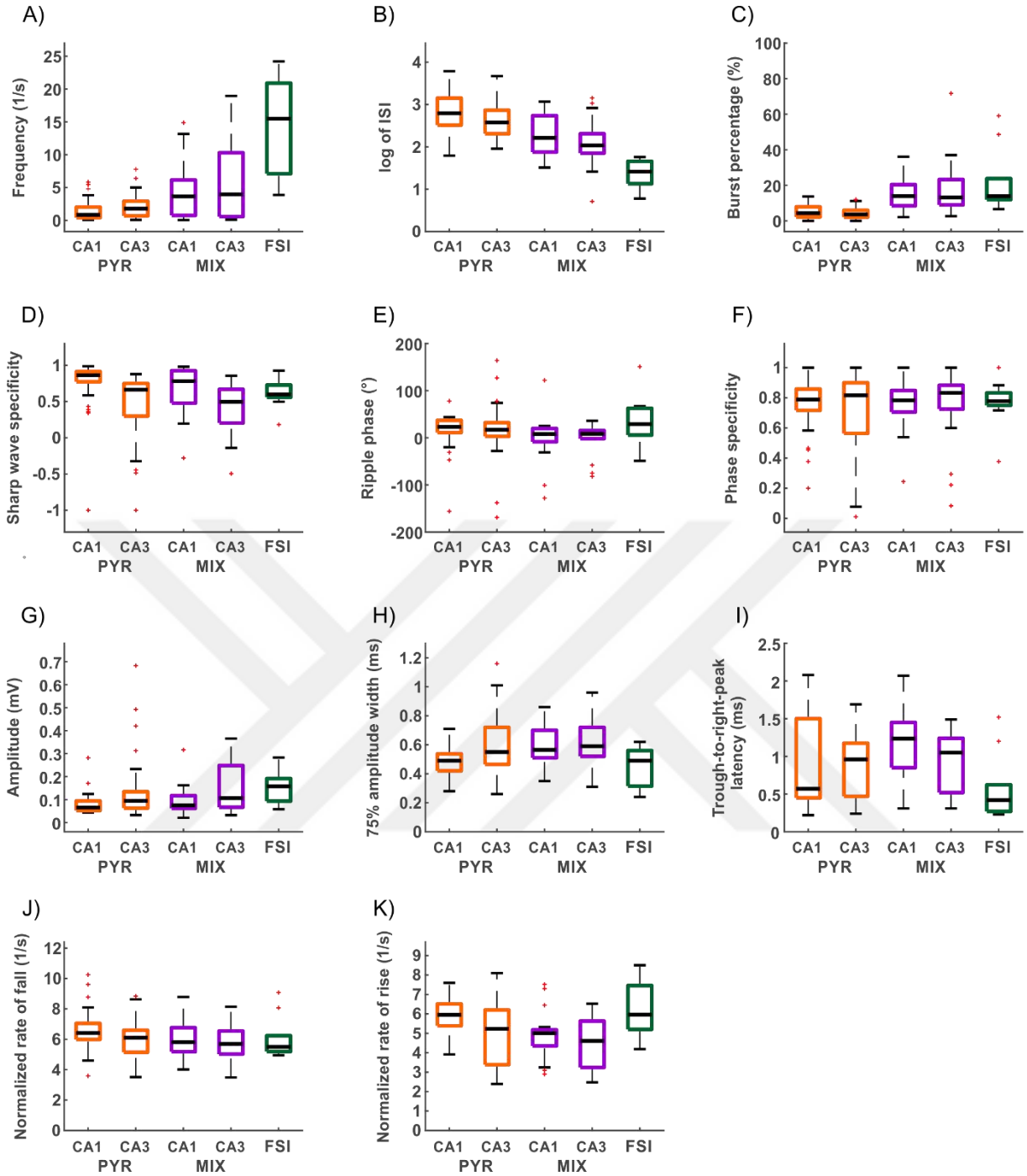


Figure 21: Baseline properties of PYR, MIX and FSI units in CA1 and CA3. A-K) Unit property boxplots of units separated into 3 classes, in all boxplots, the units shown from left to right are; Baseline PYR CA1, PYR CA3 (orange colored), Baseline MIX CA1, MIX CA3, (purple colored and Baseline FSI (green colored). FSI units were not separated into CA1 and CA3 due to their low number. ($n = 9$). **A)** Frequency, **B)** Logarithm of the inter-spike interval (ISI), **C)** Burst percentage, **D)** Sharp wave specificity, **E)** Ripple phase, **F)** Phase specificity, **G)** Amplitude, **H)** 75 % amplitude width, **I)** Trough-to-right-peak latency, **J)** Normalized rate of fall, **K)** Normalized rate of rise. $n_{PYRCA1} = 48$ units taken from 20 slices from 11 mice, $n_{PYRCA3} = 72$ units taken from 21 slices from the same 11 mice, $n_{MIXCA1} = 22$ units taken from 13 slices from the same 11 mice, $n_{MIXCA3} = 26$ units taken from 14 slices from the same 11 mice, $n_{FSI} = 9$ units taken from 8 slices from the same 11 mice.

Frequency of PYR units of the CA1 region during baseline was 0.85/s (Hz) [0.41 2.02] and frequency of PYR units of the CA3 region during baseline was 1.79/s (Hz) [0.70 2.94] (Figure 21, A). Frequency of MIX units of the CA1 region during baseline was 3.65/s (Hz) [0.76 6.15] and frequency of MIX units of the CA3 region during baseline was 3.96/s (Hz) [0.58 10.29] (Figure 21, A). Frequency of FSI units during baseline was 15.5/s (Hz) [7.11 20.90] (Figure 21, A). Logarithm of Inter-Spike Interval (log of ISI) of PYR units of the CA1 region during baseline was 2.79 [2.51 3.15] and log of ISI of PYR units of the CA3 region during baseline was 2.57 [2.31 2.86] (Figure 21, B). Log of ISI of MIX units of the CA1 region during baseline was 2.21 [1.88 2.74] and log of ISI of MIX units of the CA3 region during baseline was 2.03 [1.85 2.31] (Figure 21, B). Log of ISI of FSI units during baseline was 1.41 [1.13 1.66] (Figure 21, B). Burst Percentage of PYR units of the CA1 region during baseline was 4.33 % [2.21 7.90] and burst percentage of PYR units of the CA3 region during baseline was 3.61 % [2.08 5.97] (Figure 21, C). Burst Percentage of MIX units of the CA1 region during baseline was 13.94 % [8.47 20.39] and burst percentage of MIX units of the CA3 region during baseline was 13.16 % [8.97 23.25] (Figure 21, C). Burst Percentage of FSI units during baseline was 13.89 % [11.89 23.77] (Figure 21, C).

Sharp wave Specificity of PYR units of the CA1 region during baseline was 0.86 [0.77 0.91] and sharp wave specificity of PYR units of the CA3 region during baseline was 0.66 [0.30 .75] (Figure 21, D). Sharp wave specificity of MIX units of the CA1 region during baseline was 0.78 [0.48 0.93] and sharp wave specificity of MIX units of the CA3 region during baseline was 0.50 [0.20 0.67] (Figure 21, D). Sharp wave specificity of FSI units during baseline was 0.60 [0.56 0.73] (Figure 21, D). Median ripple phase of PYR units of the CA1 region during baseline was 23 degrees (°) [11 37] and median ripple phase of PYR units of the CA3 region during baseline was 17 degrees (°) [3 32] (Figure 21, E). Median ripple phase of MIX units of the CA1 region during baseline was 8 degrees (°) [-8.50 19.75] and median ripple phase of MIX units of the CA3 region during baseline was 8.50 degrees (°) [-2 15.50] (Figure 21, E). Median ripple phase of FSI units during baseline was 29 degrees (°) [5.75 62.50] (figure 21, E). Ripple phase specificity of PYR units of the CA1 region during baseline was 0.79 [0.72 0.86] and ripple phase specificity of PYR units of the CA3 region during baseline was 0.82 [0.56 0.90] (Figure 21, F). Ripple phase specificity of MIX units of the CA1 region during baseline was 0.78 [0.71 0.85] and unit ripple phase specificity of MIX units of the CA3 region during baseline was 0.83 [0.73 0.88] (Figure 21, F). Ripple phase specificity of FSI units during baseline was 0.78 [0.75 0.83] (Figure 21, F).

Amplitude of PYR units of the CA1 region during baseline was 0.065 mV [0.052 0.094] and amplitude of PYR units of the CA3 region during baseline was 0.095 mV [0.063 0.13] (Figure 21, G). Amplitude of MIX units of the CA1 region during baseline was 0.075 mV [0.062 0.12] and amplitude of MIX units of the CA3 region during baseline was 0.11 mV [0.067 0.25] (Figure 21, G). Amplitude of FSI units during baseline was 0.16 mV [0.093 0.19] (Figure 21, G). 75 % amplitude width (width75) of PYR units of the CA1 region during baseline was 0.49 milliseconds (ms) [0.42 0.54] and 75 % amplitude width (width75) of PYR units of the CA3 region during baseline was 0.55 milliseconds (ms) [0.47 0.72] (Figure 21, H). 75 % amplitude width (width75) of MIX units of the CA1 region during baseline was 0.57 milliseconds (ms) [0.51 0.70] and 75 % amplitude width (width75) of MIX units of the CA3 region during baseline was 0.59 milliseconds (ms) [0.52 0.72] (Figure 21, H). 75 % amplitude width (width75) of FSI units during baseline was 0.49 milliseconds (ms) [0.32 0.56] (Figure 21, H). Trough-to-right-peak latency (tRightPeak) of PYR units of the CA1 region during baseline was 0.57 milliseconds (ms) [0.45 1.50] and trough-to-right-peak latency (tRightPeak) of PYR units of the CA3 region during baseline was 0.96 milliseconds (ms) [0.47 1.18] (Figure 21, I). Trough-to-right-peak latency (tRightPeak) of MIX units of the CA1 region during baseline was 1.24 milliseconds (ms) [0.85 1.45] and trough-to-right-peak latency (tRightPeak) of MIX units of the CA3 region during baseline was 1.05 milliseconds (ms) [0.52 1.24] (Figure 21, I). Trough-to-right-peak latency (tRightPeak) of FSI units during baseline was 0.42 milliseconds (ms) [0.27 0.62] (Figure 21, I).

Normalized rate of fall (fall-norm) of PYR units of the CA1 region during baseline was 6.41/s [5.99 7.05] and normalized rate of fall (fall-norm) of PYR units of the CA3 region during baseline was 6.10/s [5.15 6.60] (Figure 21, J). Normalized rate of fall (fall-norm) of MIX units of the CA1 region during baseline was 5.81/s [5.18 6.76] and normalized rate of fall (fall-norm) of MIX units of the CA3 region during baseline was 5.70/s [5.03 6.55] (Figure 21, J). Normalized rate of fall (fall-norm) FSI units during baseline was 5.50/s [5.19 6.23] (Figure 21, J). Normalized rate of rise (rise-norm) of PYR units of the CA1 region during baseline was 5.95/s [5.39 6.51] and normalized rate of rise (rise-norm) of PYR units of the CA3 region during baseline was 5.23/s [3.39 6.20] (Figure 21, K). Normalized rate of rise (rise-norm) of MIX units of the CA1 region during baseline was 5.01/s [4.35 5.18] and normalized rate of rise (rise-norm) of MIX units of the CA3 region during baseline was 4.61/s [3.24 5.63] (Figure 21, K). Normalized rate of rise (rise-norm) of FSI units during baseline was 5.96/s [5.20 7.46] (Figure 21, K).

3.1.2.1. One hour post SD properties of PYR, MIX and FSI units

Unit frequency of MIX was significantly reduced for both CA1 and CA3 regions at one hour post SD unlike PYR frequency which didn't show significant change

As it was done for one hours post SD values of the previous sections, since the PYR, MIX and FSI units of OGD 1h slices are the same ones as baseline units due to the continuous tetrode recordings, individual values are shown on the boxplots with lines connecting each baseline value to the corresponding one hour post SD value for figures 22, 23 and 24. Due to the small number of baseline FSI units of the OGD slices (n=2) boxplots could not be plotted for FSI comparing baseline and one hour post SD values and no statistical analysis was made. The two units are shown as dots with blue lines connecting baseline values to their corresponding one hours post SD values (Figure 24).

As discussed in the one hour post SD unseparated units section, looking at the unit frequency boxplots with the connected lines of each value it was observed that units with higher baseline frequency seemed to show a clearer reduction of frequency at one hour post SD than units with lower baseline frequency (Figure 17, A). The analysis of separated unit confirms this observation. The PYR units didn't show a reduction in frequency (Figure 22, A), while there was a significant reduction of frequency for the MIX units in both CA1 ($p=0.0026$) and CA3 ($p=0.0061$) regions (Figure 23, A).

Again looking at the MIX boxplots comparing baseline and one hour post SD values (Figure 23,A) it can be seen that MIX cells that had a lower baseline frequency stayed more or less at the same frequency whereas MIX cells with higher baseline frequencies were reduced, again suggesting that fast-spiking interneurons are the ones affected by the mild OGD. Looking at the 2 FSI units, the unit frequency of these units also was reduced at one hour post SD, (Figure 24, A) so there seems to be a tendency for FSI units to reduce in frequency following the initial one hour recovery from mild OGD.

Sharp wave specificity (unit to SPW-R coupling) of all units of the CA1 region seemed to have increased at one hour post SD which was shown in Figure 17 (Figure 17, A). Looking at the separated unit's boxplots, it can be seen that for the PYR units, there is no such increase, (Figure 22, C) but there is an almost significant increase for the MIX units of the CA1 region from baseline to one hour post SD ($p=0.055$) (Figure 23, C) and the two FSI units both show an increase in sharp wave specificity (Figure 24, C).

For the PYR units there was no significant change in burst percentage (Figure 22, B), sharp wave specificity (Figure 22, C), median ripple phase where units fired (Figure 22, D), ripple phase specificity (Figure 22, E), amplitude (Figure 22, F), 75 % amplitude width (width75) (Figure 22, G), normalized rate of rise (rise-norm) (Figure 22, I) and trough-to-right-peak latency (tRighrPeak) (Figure 22, J) from baseline to one hour post SD for both the CA1 and CA3 regions. There was a significant increase in the normalized rate of fall (fall-norm) ($p=4.27 \times 10^{-4}$), from baseline to one hour post SD, for the PYR units of the CA1 region, there was no significant change for the PYR units of the CA3 region (Figure 22, H).

For the MIX units there was no significant change in burst percentage (Figure 23, B), sharp wave specificity (Figure 23, C), median ripple phase where units fired (Figure 23, D), ripple phase specificity (Figure 23, E), amplitude (Figure 23, F), normalized rate of fall (fall-norm) (Figure 23, H) and trough-to-right-peak latency (tRighrPeak) (Figure 23, J) from baseline to one hour post SD for both the CA1 and CA3 regions. There was a significant decrease in the normalized rate of rise (rise-norm) ($p=0.034$), from baseline to one hour post SD, for the MIX units of the CA1 region, but not for the MIX units of the CA3 region (Figure 23, I). There was a significant increase in the 75 % amplitude width (width75) ($p=0.0029$), from baseline to one hour post SD for the MIX units of the CA3 region, but not for the MIX units of the CA3 region (Figure 23, G),

One of the two baseline FSI units of the OGD slices, did not have a detectable median ripple phase (Figure 24, D) or ripple phase specificity (Figure 24, E) at one hour post SD. The trough-to-right-peak latency (tRightPeak) of both FSI units increased from baseline to one hour post SD (Figure 24, J). There was no such uniform change in burst percentage (Figure 24, B), amplitude, (Figure 24, F), 75 % amplitude width (width 75) (Figure 24, G), normalized rate of fall (fall-norm) (Figure 24, H) and normalized rate of rise (rise-norm) (Figure 24, I) from baseline to one hour post SD for the two FSI units.

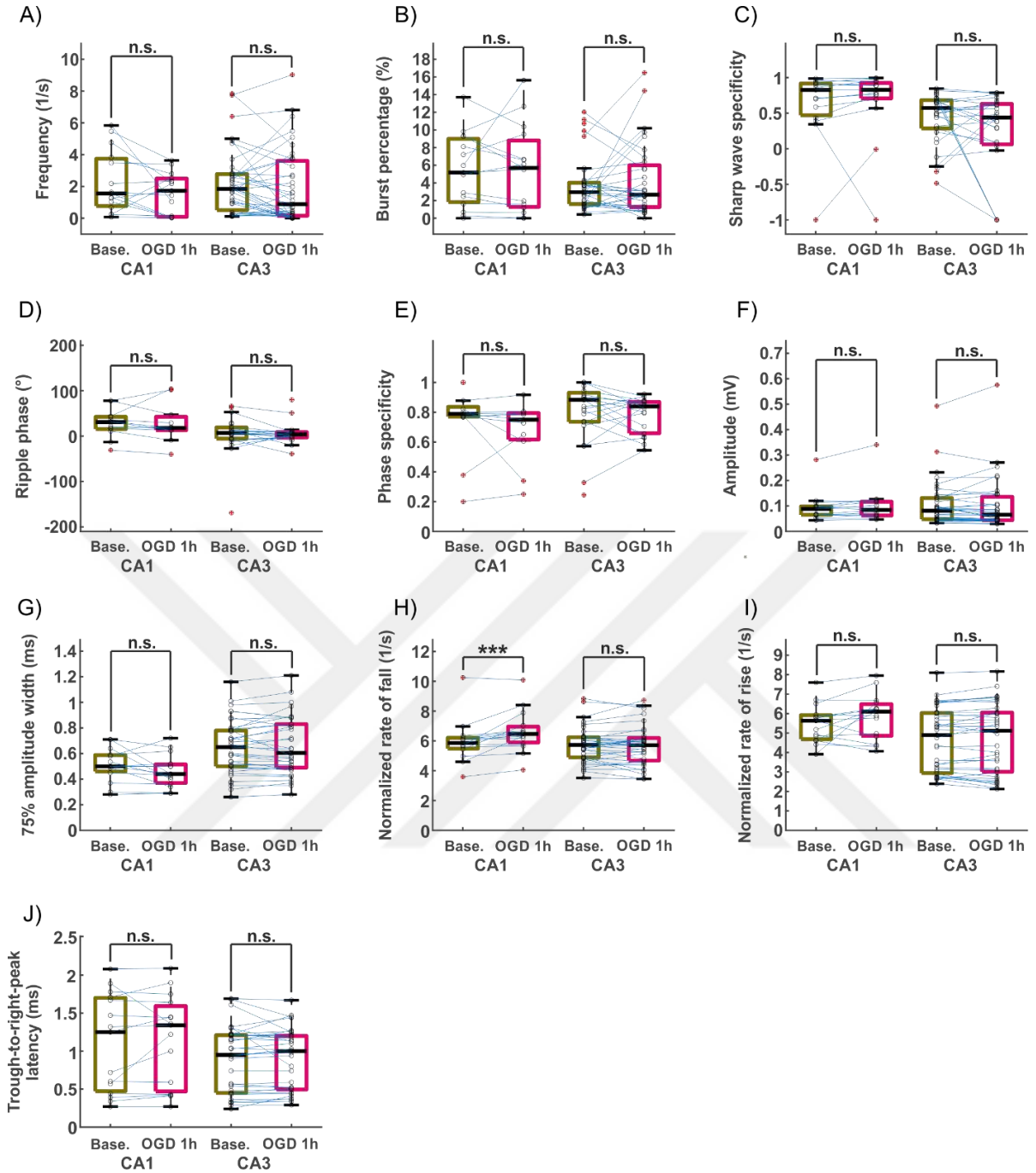


Figure 22: Properties of PYR units in CA1 and CA3 during baseline and one hour post SD. A-K) Unit property boxplots of PYR units comparing Baseline and OGD 1hour conditions for both CA1 and CA3 regions, in all boxplots, the units shown from left to right are; PYR CA1 Baseline, (light green colored), PYR CA1 OGD 1hour (pink colored), PYR CA3 Baseline (light green colored), PYR CA3 OGD 1hour (pink colored). All p values are calculated using the Wilcoxon signed-rank test. **A)** Frequency, $p_{CA1} = 0.17$, $p_{CA3} = 0.26$, **B)** Burst percentage, $p_{CA1} = 0.64$, $p_{CA3} = 0.85$, **C)** Sharp wave specificity, $p_{CA1} = 0.12$, $p_{CA3} = 0.31$, **D)** Ripple phase, $p_{CA1} = 0.32$, $p_{CA3} = 0.17$, **E)** Phase specificity, $p_{CA1} = 0.98$, $p_{CA3} = 0.51$, **F)** Amplitude, $p_{CA1} = 0.21$, $p_{CA3} = 0.59$, **G)** 75 % amplitude width, $p_{CA1} = 0.11$, $p_{CA3} = 0.67$, **H)** Normalized rate of fall, $p_{CA1} = 4.27 \times 10^{-4}$, $p_{CA3} = 0.83$, **I)** Normalized rate of rise, $p_{CA1} = 0.21$, $p_{CA3} = 0.65$, **J)** Trough-to-right-peak latency, $p_{CA1} = 0.33$, $p_{CA3} = 0.24$, $n_{PYRCA1} = 15$ units taken from 8 slices from 11 mice, $n_{PYRCA3} = 39$ units taken from 11 slices from the same 11 mice

Base. = Baseline, n.s. = not significant, ***= $p < 0.001$, p = p value

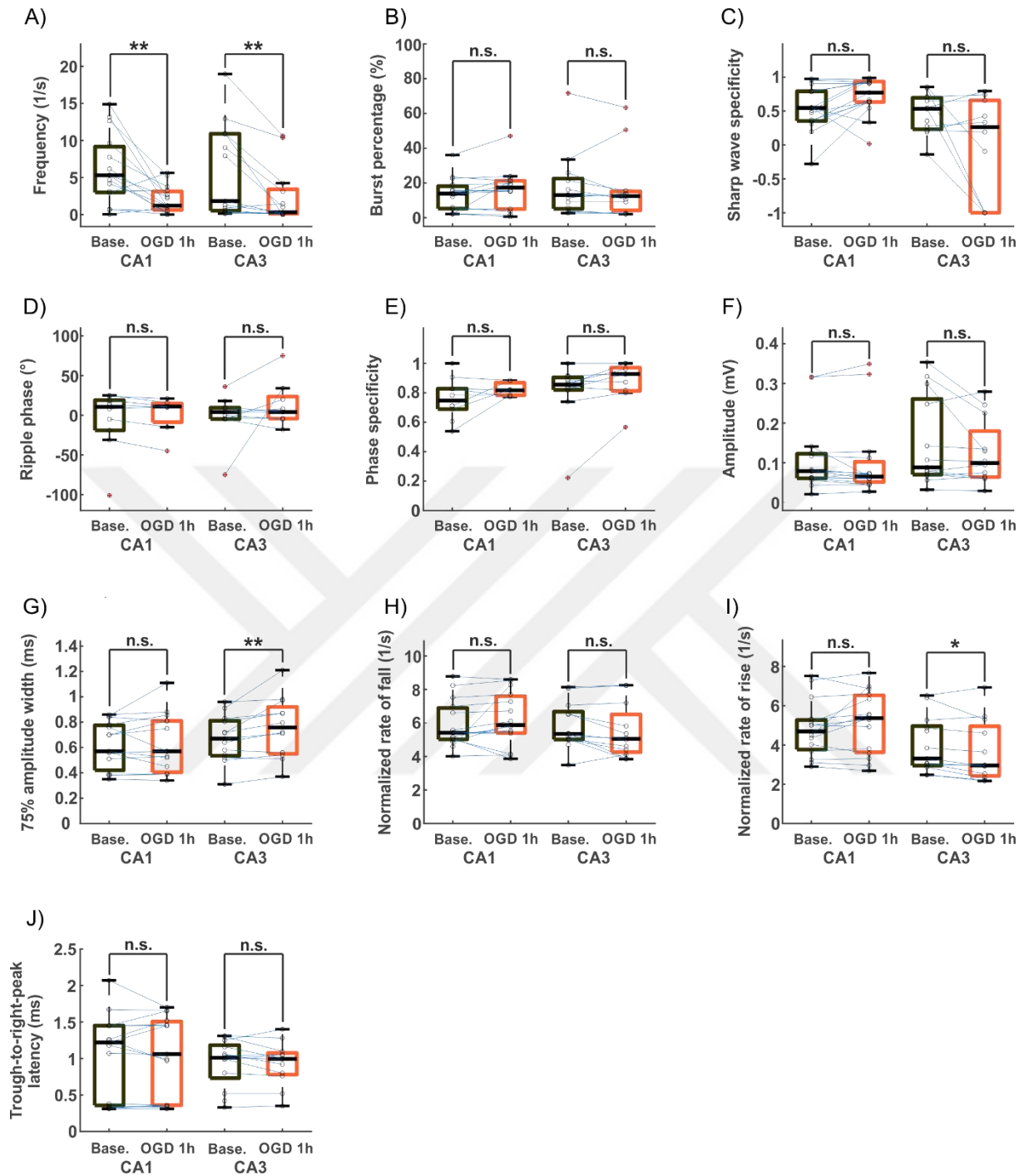


Figure 23: Properties of MIX units in CA1 and CA3 during baseline and one hour post SD. A-K) Unit property boxplots of MIX units comparing Baseline and OGD 1hour conditions for both CA1 and CA3 regions, in all boxplots, the units shown from left to right are; MIX CA1 Baseline, (dark green colored), MIX CA1 OGD 1hour (salmon colored), MIX CA3 Baseline (dark green colored), MIX CA3 OGD 1hour (salmon colored). All p values are calculated using the Wilcoxon signed-rank test. **A)** Frequency, $p_{CA1} = 0.0026$, $p_{CA3} = 0.0061$, **B)** Burst percentage, $p_{CA1} = 0.33$, $p_{CA3} = 0.24$, **C)** Sharp wave specificity, $p_{CA1} = 0.055$, $p_{CA3} = 0.43$, **D)** Ripple phase, $p_{CA1} = 0.063$, $p_{CA3} = 0.47$, **E)** Phase specificity, $p_{CA1} = 0.44$, $p_{CA3} = 0.063$, **F)** Amplitude, $p_{CA1} = 0.39$, $p_{CA3} = 0.27$, **G)** 75 % amplitude width, $p_{CA1} = 0.55$, $p_{CA3} = 0.0029$, **H)** Normalized rate of fall, $p_{CA1} = 0.15$, $p_{CA3} = 0.092$, **I)** Normalized rate of rise, $p_{CA1} =$

0.14, $p_{CA3} = 0.034$, **J**) Trough-to-right-peak latency, $p_{CA1} = 0.99$, $p_{CA3} = 0.18$, $n_{MIXCA1} = 15$ units taken from 8 slices from 11 mice, $n_{MIXCA3} = 13$ units taken from 8 slices from the same 11 mice

Base. = Baseline, n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, p = p value

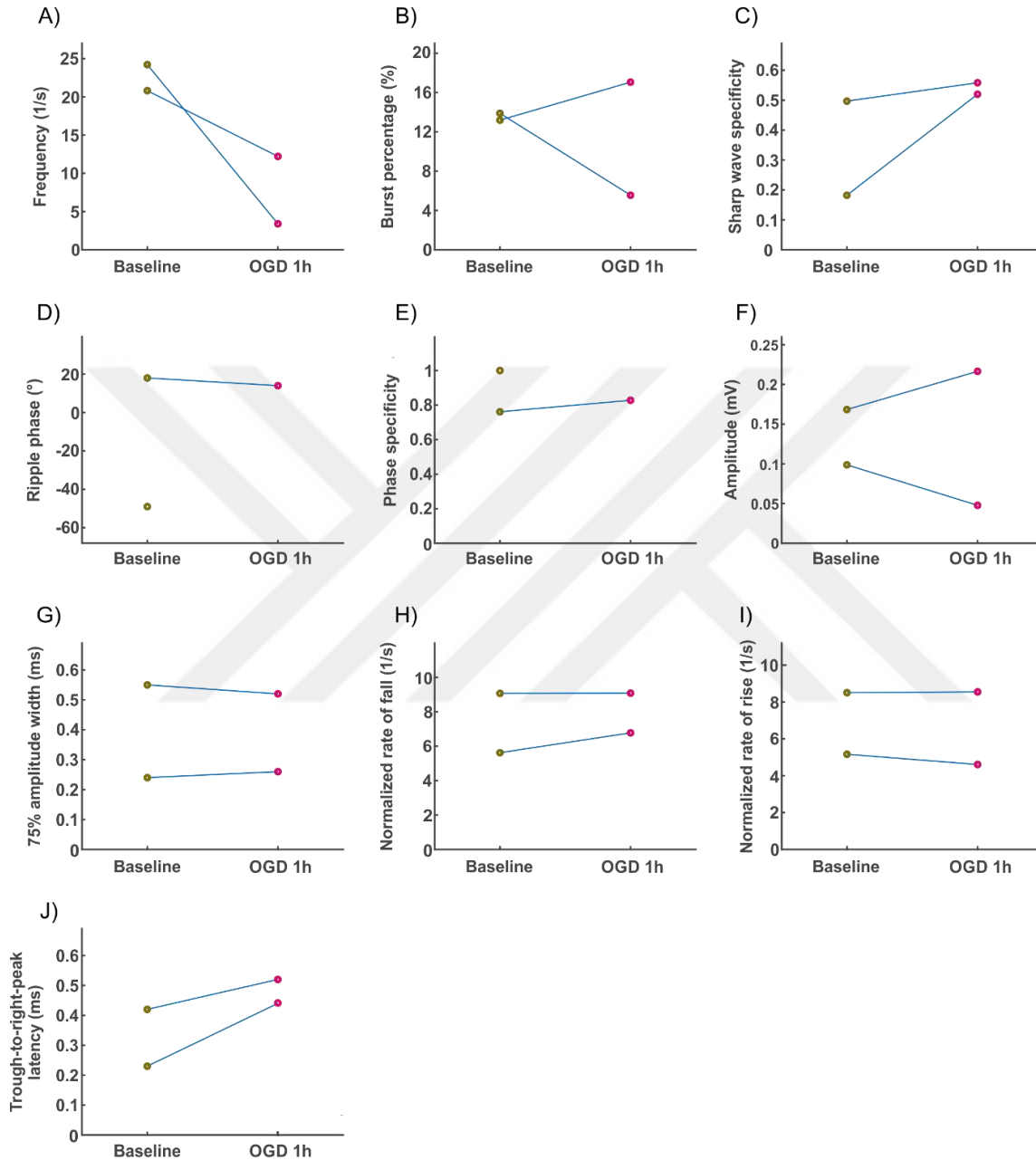


Figure 24: Properties of FSI units in CA1 and CA3 during baseline and one hour post SD. A-K) Unit property plots of FSI units comparing Baseline and OGD 1hour conditions. Boxplots and statistical analysis of these units were not possible due to the low number. ($n = 2$). Therefore, the values themselves are plotted on the graphs as points and connected by blue lines to emphasize the change from baseline to OGD 1hour. In all plots, the values shown from left to right are; FSI baseline, (light green colored circles and FSI OGD 1hour (pink colored circles). **A)** Frequency, **B)** Burst percentage, **C)** Sharp wave specificity, **D)** Ripple phase, **E)** Phase specificity, **F)** Amplitude, **G)** 75 % amplitude width, **H)** Normalized rate of fall, **I)** Normalized rate of rise, **J)** Trough-to-right-peak latency, $n_{FSI} = 2$ units from 2 slices from 11 mice.

3.1.2.2. Four hours post SD properties of PYR, MIX and FSI units

PYR firing rate was higher for OGD 4h slices compared to control 4h slices for both CA1 and CA3 regions, FSI firing rate was lower and the MIX sharp wave specificity was lower for OGD 4h slices compared to control 4h slices for the CA1 region

For the OGD 4h and control 4h slice comparison boxplots of separated units, there are no single values shown and there are no lines connecting the boxplots since these units are completely different from units recorded from baseline to one hour post SD. Hence, single unit activity values can only be compared between OGD and control with no pairing of values or normalization to baseline possible.

In the unseparated unit activity results, comparing OGD 4h and control 4h slices the unit frequency of OGD 4h slices were significantly higher for the CA3 region ($p=0.016$) (Figure 20, A). This result was unexpected but it should be noted that no previous study looked at four hours recovery post SD. Looking at the separated unit plots, it can be seen that the PYR of OGD 4h slices were significantly higher compared to control 4h slices for both CA1 ($p=0.043$) and CA3 ($p=0.033$) regions (Figure 25, A). The log of ISI of PYR units of the OGD 4h slices were also significantly smaller compared to control 4h slices for the CA3 region ($p=0.025$) and almost significantly smaller for the CA1 region ($p=0.054$) (Figure 25, B), showing additional evidence that pyramidal cells of OGD 4h slices fired more frequently with smaller inter-spike intervals than the pyramidal cells of control 4h slices.

The sharp wave specificity of PYR units of the OGD 4h slices were almost significantly smaller compared to control 4h slices for the CA3 region ($p=0.06$) and the CA1 region also showed a tendency to be smaller although it was also an insignificant change ($p=0.15$) (Figure 25, D). The MIX units showed no significant difference in frequency between OGD 4h and control 4h slices for both CA1 and CA3 regions (Figure 26, A), however, the logarithm of inter-spike interval (log of ISI) of MIX units of the OGD 4h slices were significantly smaller compared to control 4h slices for the CA1 region ($p=0.02$) (Figure 26, B). The sharp wave specificity of the MIX units of the OGD 4h slices were significantly smaller compared to control 4h slices for the CA1 region ($p=0.017$) (Figure 26,D). This tendency to have less unit to SPW-R coupling of both PYR and MIX units four hours after SD again suggests that as PYR cells fire more frequently following mild OGD, the spikes tend to become less coupled to SPW-R which

increases the risk of pyramidal cells firing with less precision and the neuronal network of the hippocampus to lose its temporal coding ability.

There was only 5 FSI from OGD 4h slices and 6 FSI from control 4h slices so these units were not separated into CA1 and CA3 regions and were pooled together (Figure 27). The unit frequency of OGD 4h FSI is almost significantly lower than control 4h FSI ($p=0.052$) (Figure 27, A). This shows that following mild OGD, after one hour recovery, PYR units fire with no change to their baseline frequency (Figure 22, A) and FSI units have a tendency to fire with lower frequency (Figure 24, A) and after four hours recovery the tendency for FSI units to fire less frequently persist but the PYR units fire at a higher frequency compared to control slices (Figure 25, A) possibly due to disinhibition.

For the PYR units, the amplitude of the OGD 4h units was significantly smaller compared to control 4h PYR units for both the CA1 ($p=4.09 \times 10^{-4}$) and the CA3 ($p=0.0045$) regions (Figure 25, G). The 75 % amplitude width (width75) of the OGD 4h PYR units was significantly larger compared to control 4h PYR units for the CA3 region ($p=0.013$) but not for the CA1 region. The normalized rate of fall (fall-norm) of OGD 4h PYR units was significantly smaller compared to control 4h PYR units for the CA3 region ($p=0.038$) but not for the CA1 region (Figure 25, J) and this was also true for the normalized rate of rise (rise-norm) ($p_{CA3} = 0.016$) (Figure 25, K). There was no significant difference in burst percentage (Figure 25, C), median ripple phase where units fired (Figure 25, E), ripple phase specificity (Figure 25, F) and trough-to-right-peak latency (tRight-Peak) (Figure 25, I) between OGD 4h and control 4h PYR units for both CA1 and CA3 regions.

For the MIX units, the amplitude of the OGD 4h units was significantly smaller compared to control 4h MIX units for the CA3 region ($p=0.035$) but not for the CA1 region (Figure 26, G). The 75 % amplitude width (width 75) of the OGD 4h MIX units was significantly larger than the control 4h MIX units for the CA1 region ($p=0.0039$) but not for the CA3 region (Figure 26, H). The normalized rate of rise (rise-norm) of the OGD 4h MIX units was significantly smaller than the control 4h MIX units for the CA1 region ($p=0.032$) but not for the CA3 region (Figure 26, K). There was no significant difference in burst percentage (Figure 26, C), median ripple phase where units fired (Figure 26, E), ripple phase specificity (Figure 26, F) trough-to-right-peak latency (tRight-Peak) (Figure 26, I) and normalized rate of fall (fall-norm) (Figure 26, J) between OGD 4h and control 4h MIX units for both CA1 and CA3 regions.

For the FSI units, there was no significant difference in logarithm of inter-spike interval (log of ISI), burst percentage (Figure 27, C), sharp wave specificity (Figure 27, D), median ripple phase where units fired (Figure 27, E), ripple phase specificity (Figure 27, F), amplitude (Figure 27, G), 75 % amplitude width (width 75) (Figure 27, H), trough-to-right-peak latency (tRight-Peak) (Figure 27, I), normalized rate of fall (fall-norm) (Figure 27, J) and normalized rate of rise (Figure 27, K), between OGD 4h and control 4h units.

The finding of increased firing frequency of PYR units four hours post SD along with the finding of a decrease in frequency of FSI units, suggests that as time goes on following mild OGD, this effect will continue, possibly getting stronger along with the manifestation of other effects caused by the disruption of mild OGD. So a longer recovery period, such as 8 hours, or even 24 hours would possibly yield more significant results. This is why it is important to find a method to increase the viability of acute hippocampal slices in the Hass-type interface chamber. 21-hour UVPS experiment results shows promise in this regard.

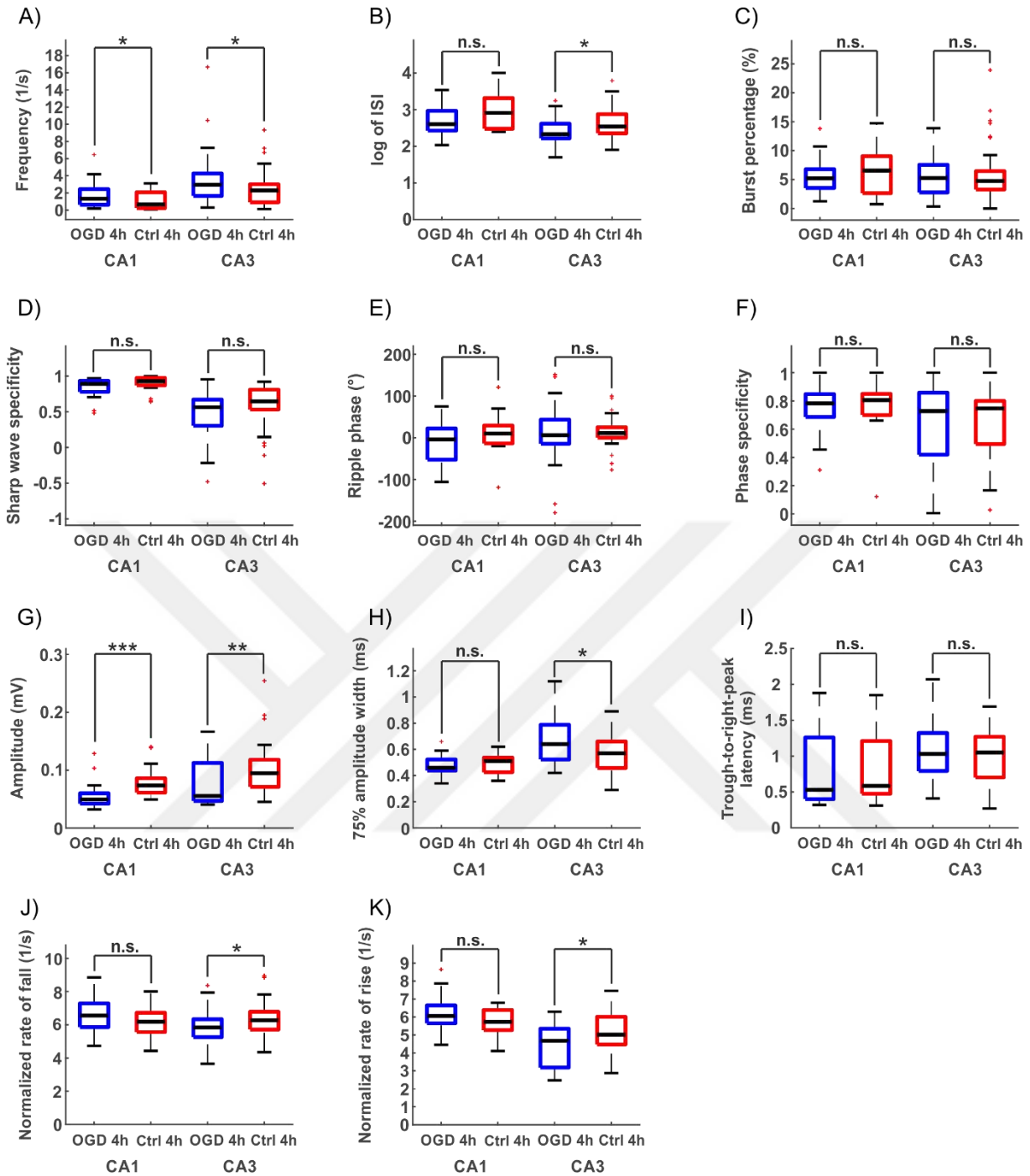


Figure 25: Properties of PYR units in CA1 and CA3, comparing OGD and control slices four hours post SD. A-K) Unit property boxplots of PYR units, comparing OGD4h and control 4h, for both CA1 and CA3 regions. In all boxplots, the units shown from left to right are; PYR CA1 OGD 4hour (blue colored), PYR CA1 control 4hour (red colored), PYR CA3 OGD 4hour (blue colored), PYR CA3 control 4hour (red colored). All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. (A) Frequency, $p_{CA1} = 0.043$, $p_{CA3} = 0.033$, (B) Logarithm of the inter-spike interval (ISI), $p_{CA1} = 0.054$, $p_{CA3} = 0.025$, (C) Burst percentage, $p_{CA1} = 0.56$, $p_{CA3} = 0.90$, (D) Sharp wave specificity, $p_{CA1} = 0.15$, $p_{CA3} = 0.060$, (E) Ripple phase, $p_{CA1} = 0.28$, $p_{CA3} = 0.67$, (F) Phase specificity, $p_{CA1} = 0.73$, $p_{CA3} = 0.78$, (G) Amplitude, $p_{CA1} = 4.09 \times 10^{-4}$, $p_{CA3} = 0.0045$, (H) 75 % amplitude width, $p_{CA1} = 0.54$, $p_{CA3} = 0.013$, (I) Trough-to-right-peak latency, $p_{CA1} = 0.67$, $p_{CA3} = 0.75$, (J) Normalized rate of fall, $p_{CA1} = 0.17$, $p_{CA3} = 0.038$, (K) Normalized rate of rise, $p_{CA1} = 0.20$, $p_{CA3} = 0.016$, $n_{OGD4hCA1} = 21$ units taken

from 8 slices from 11 mice, $n_{\text{Ctrl4hCA1}} = 20$ units taken from 10 slices from 11 mice, $n_{\text{OGD4hCA3}} = 39$ units taken from 10 slices from the same 11 mice, $n_{\text{Ctrl4hCA3}} = 39$ units taken from 12 slices from the same 11 mice.

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, p = p value, Ctrl = control

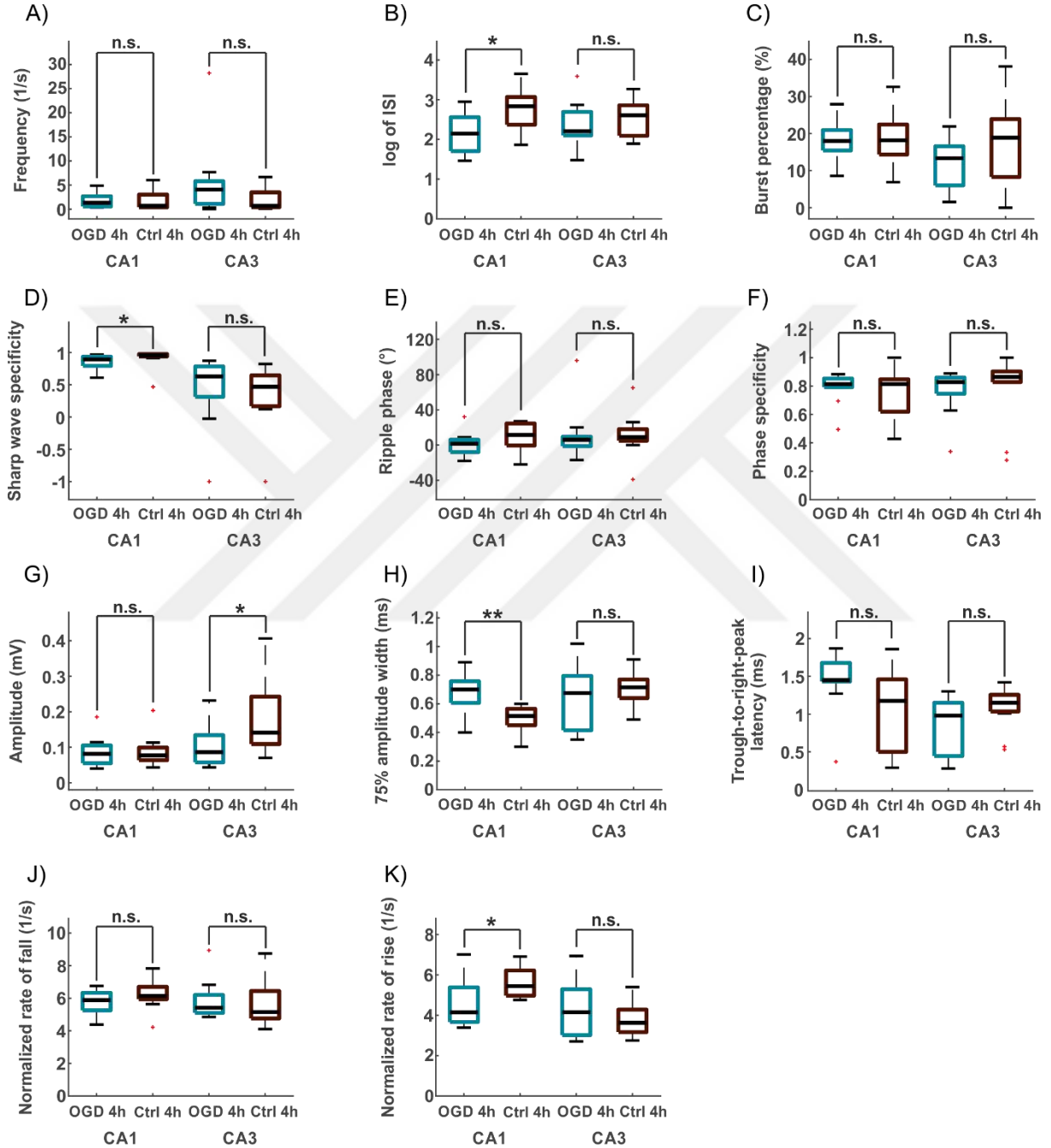


Figure 26: Properties of MIX units in CA1 and CA3, comparing OGD and control slices four hours post SD. A-K) Unit property boxplots of MIX units, comparing OGD 4 h and control 4h, for both CA1 and CA3 regions. In all boxplots, the units shown from left to right are; MIX CA1 OGD 4hour (light blue colored), MIX CA1 control 4hour (brown colored), MIX CA3 OGD 4hour (light blue colored), and MIX CA3 control 4hour (brown colored). All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. **(A)** Frequency, $p_{\text{CA1}} = 0.64$, $p_{\text{CA3}} = 0.11$. **(B)** Logarithm of the inter-

spike interval (ISI), $p_{CA1} = 0.02$, $p_{CA3} = 0.37$. **C)** Burst percentage, $p_{CA1} = 0.93$, $p_{CA3} = 0.13$. **D)** Sharp wave specificity, $p_{CA1} = 0.017$, $p_{CA3} = 0.39$. **E)** Ripple phase, $p_{CA1} = 0.15$, $p_{CA3} = 0.36$. **F)** Phase specificity, $p_{CA1} = 0.56$, $p_{CA3} = 0.17$. **G)** Amplitude, $p_{CA1} = 0.83$, $p_{CA3} = 0.035$. **H)** 75 % amplitude width, $p_{CA1} = 0.0039$, $p_{CA3} = 0.60$. **I)** Trough-to-right-peak latency, $p_{CA1} = 0.077$, $p_{CA3} = 0.064$. **J)** Normalized rate of fall, $p_{CA1} = 0.15$, $p_{CA3} = 0.54$. **K)** Normalized rate of rise, $p_{CA1} = 0.032$, $p_{CA3} = 0.58$. $n_{OGD4hCA1} = 13$ units taken from 9 slices from 11 mice, $n_{Ctrl4hCA1} = 10$ units taken from 6 slices from the same 11 mice, $n_{OGD4hCA3} = 12$ units taken from 9 slices from the same 11 mice, $n_{Ctrl4hCA3} = 12$ units taken from 8 slices from the same 11 mice.

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, p = p value, Ctrl = control

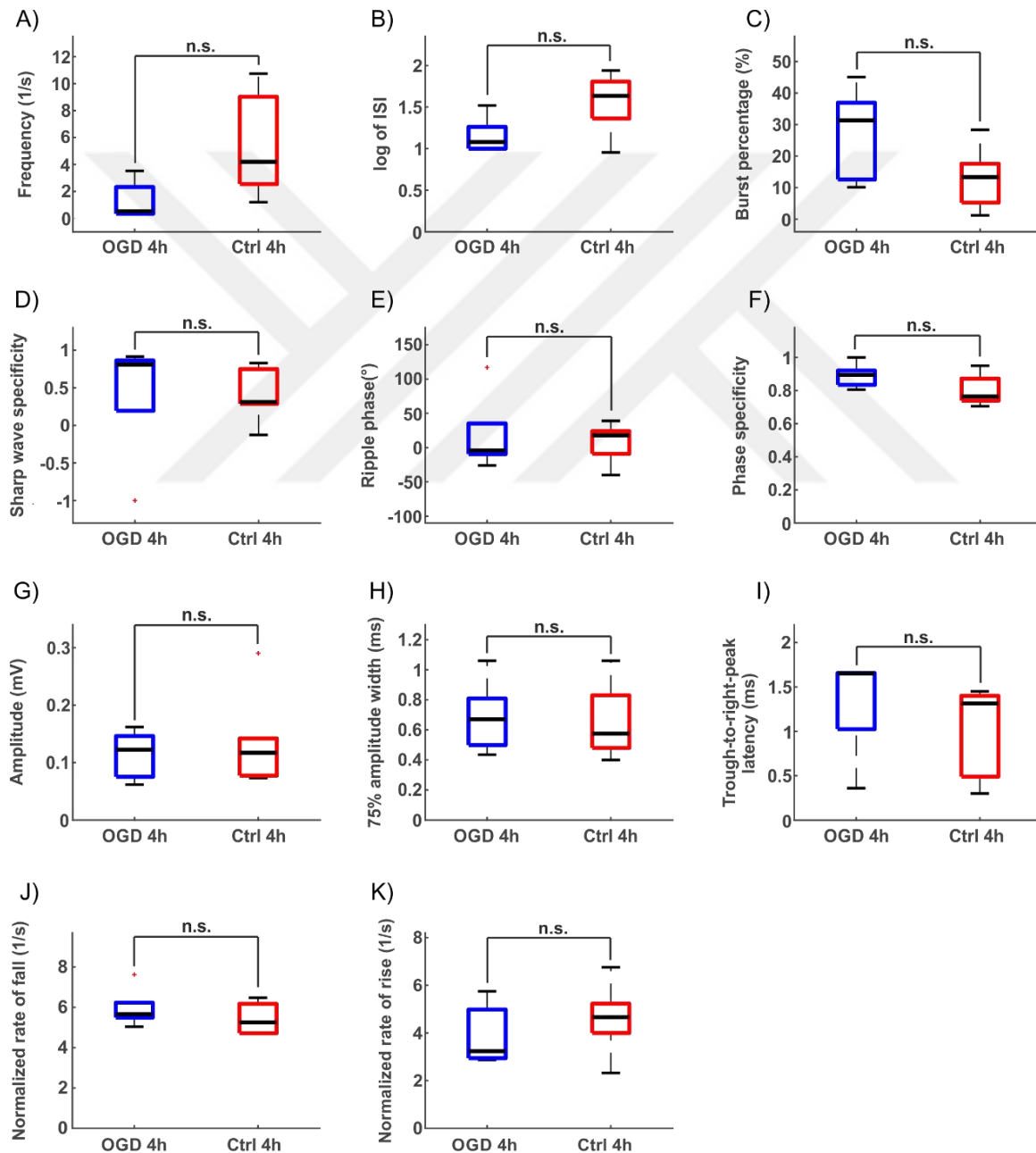


Figure 27: Properties of FSI units in CA1 and CA3, comparing OGD and control slices four hours post SD. A-K) Unit property boxplots of FSI units, comparing OGD 4h and control 4h, the units are not separated into CA1 and CA3 regions due to low number of units. (Total number of units for OGD

and control 4h combined is 11). In all boxplots, the units shown from left to right are; FSI OGD 4hour (blue colored), FSI control 4hour (red colored). All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test, unless specified otherwise. **(A)** Frequency, $p = 0.052$. **(B)** Logarithm of the inter-spike interval (ISI), $p = 0.16$. **(C)** Burst percentage, $p = 0.18$. **(D)** Sharp wave specificity, $p = 0.92$ (unpaired Student's t-test). **(E)** Ripple phase, $p = 0.76$. **(F)** Phase specificity, $p = 0.11$. **(G)** Amplitude, $p = 0.93$. **(H)** 75 % amplitude width, $p = 0.77$. **(I)** Trough-to-right-peak latency, $p = 0.30$. **(J)** Normalized rate of fall, $p = 0.33$. **(K)** Normalized rate of rise, $p = 0.66$. $n_{\text{OGD4hFSI}} = 5$ units taken from 4 slices from 11 mice, $n_{\text{Ctrl4hFSI}} = 6$ units taken from 5 slices from the same 11 mice

n.s. = not significant, p = p value, Ctrl = control

3.1.3. 21-hour UVPS of P60-70 mice

Only the CA3 region of UVPS slices show SPW-R activity after 21 hours of incubation with reduced ripple energy

As explained in the methods section, the 21-hour UVPS (ultraviolet + penicillin + streptomycin) was inspired by a previous paper which used a submerged chamber that is connected to a separate UV light containing chamber named 'Braincubator' to incubate acute brain slices for up to 36 hours (Buskila et al., 2014). In this paper, the viability of acute hippocampal brain slices were measured partly by recording the spontaneous activity and induced membrane properties of layer V pyramidal neurons that project from the cerebral cortex onto subcortical areas. However, there was no network oscillation recordings taken of these slices (Buskila et al., 2014). In the 21-hour UVPS method, CA1 and CA3 pyramidal cell layer network oscillation recordings were possible through tetrodes since these slices were incubated in a Haas-type interface chamber. These 10 minute recordings were taken during baseline (2 hours after slices were put into their respective interface chambers) and during 21-hour (21 hours after slices were put into their respective interface chambers).

The UVPS experiment was performed on 5 UVPS and control slice pairs from 2 mice on 2 different days, however, the 2 slice pairs of one of the mice showed no significant network activity after 21 hours in both UVPS and control slices so the electrophysiological data of these 2 slice pairs are not shown. However, the histology results of these 2 slice pairs were taken into account and will be shown in the histology section. 3 UVPS slices of 1 mice showed significant network activity for the CA3 region after 21 hours incubation, but no significant network activity for the CA1 region was observed (Figure 28, bottom left). In figure 28, 5 seconds example traces of baseline and 21 hour tetrode recordings can be seen. The UVPS and control slices showed similar network activity during baseline condition, however, at 21 hour, only the CA3 region of the UVPS slices had significant SPW-R activity.

Since there were only 3 UVPS and control slice pairs available, no statistical analysis could be performed and no boxplots can be plotted so the network oscillation properties of these slices were plotted as dots. Blue lines were plotted that connect baseline value of one slice to their respective 21-hour value just to make the change in value clearer to the reader for these 3 slices, even though these values are not paired (Figure 29). CA1 region of 2 UVPS and 1 control slice at 21 hours were able to be analyzed by the MATLAB script even though there was almost no SPW-R activity for these regions so when applicable these values are shown as dots.

Looking at the dotted plots, it can be seen that the sharp wave frequency of any region except the UVPS CA3, goes to 0 or almost 0 at 21-hours (Figure 29, A). Sharp wave amplitude of 2 of the 3 UVPS CA3 regions were reduced at 21-hours (Figure 29, B). Sharp wave width of the CA3 region of the UVPS slices stayed relatively same from baseline to 21 hours incubation (Figure 29, C) and two of the CA3 regions of UVPS slices showed a decrease in MUA frequency and one showed an increase from baseline to 21 hour incubation (Figure 29, F).

For the ripple frequency and ripple energy plots, only the CA3 region of the UVPS slices have dots at 21-hours because other regions had no significant ripple activity that could have been analyzed (ripple energy median of the 2 UVPS and 1 control CA1 region at 21-hours were less than 3 and were discarded). (Figure 29, D, E). Ripple energy of all 3 UVPS CA3 regions were reduced at 21-hours (Figure 29, E). Looking at example traces of a single SPW-R complex of the UVPS CA3 regions recorded during baseline (Figure 29, G, left column), and during 21-hours (Figure 29, G, right column) it can also be seen that the sharp wave amplitude and ripples of the SPW-R complexes seems to be disrupted at 21-hours especially for slices 2 and 3.

Overall these results show that for the baseline conditions UVPS treatment of acute brain slices doesn't lead to any unwanted SPW-R disruptions such as epilepsy or a reduction of SPW-R activity at least for the low penicillin and streptomycin concentrations that were used in this method.(0.042 mM and 0.043 mM respectively) The UVPS treatment showed a benefit in maintaining CA3 SPW-R activity after 21-hours of incubation even though ripple energy was reduced and 2 of the 3 slices had smaller sharp wave amplitude at 21-hours.

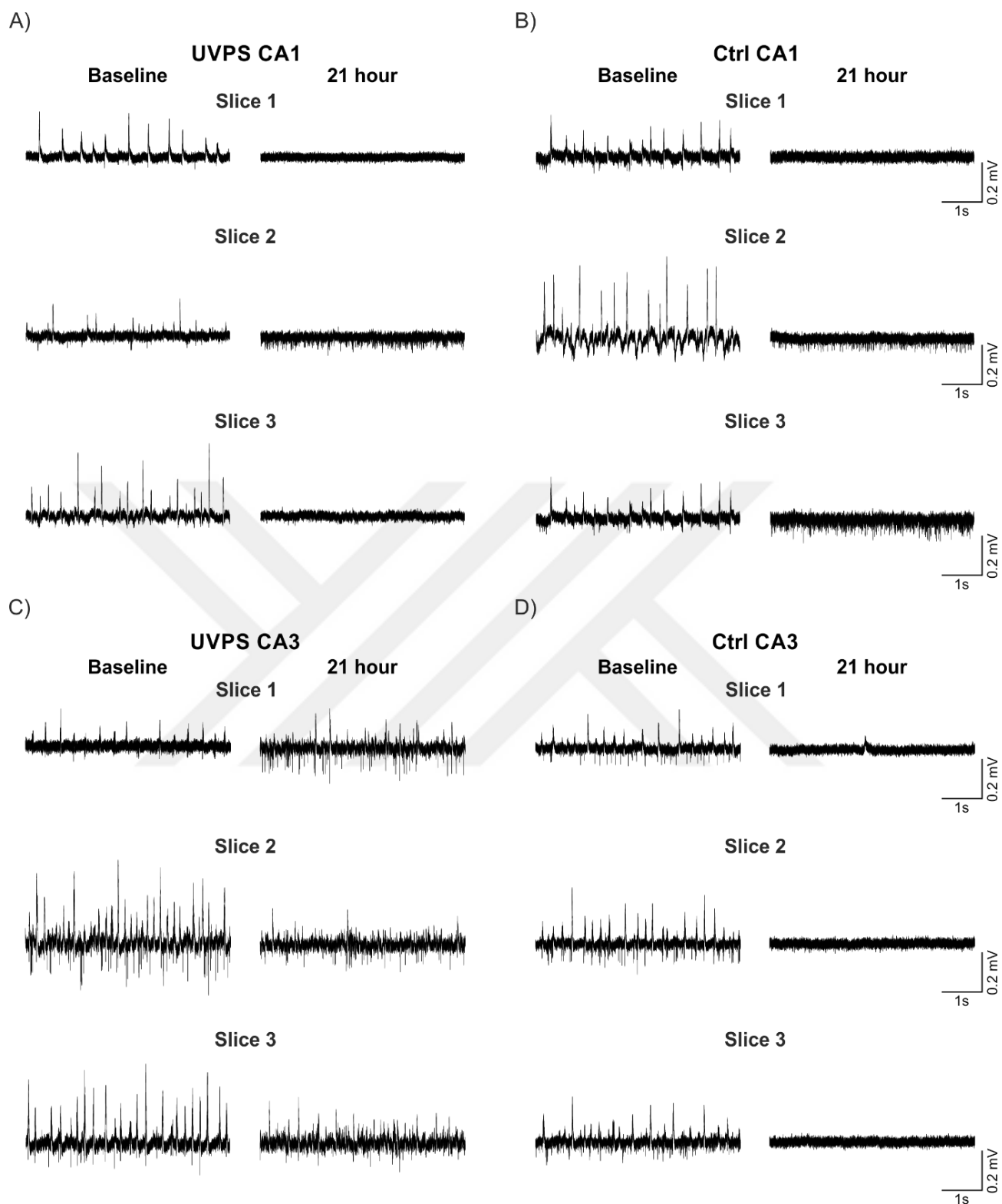


Figure 28: Example traces of tetrode recordings taken from CA1 and CA3 stratum pyramidale of UVPS and control slices taken at baseline and at 21 hour incubation. A) Example 5 second traces of UVPS CA1 tetrode recordings taken at baseline (left column) and 21-hour (right column) conditions. Slices 1 to 3 are shown from top to bottom. **B)** Example 5 second traces of control CA1 tetrode recordings taken at baseline (left column) and 21-hour (right column) conditions. Slices 1 to 3 are shown from top to bottom. **C)** Example 5 second traces of UVPS CA3 tetrode recordings taken at baseline (left column) and 21-hour (right column) conditions. Slices 1 to 3 are shown from top to bottom. **D)** Example

5 second traces of control CA3 tetraode recordings taken at baseline (left column) and 21-hour (right column) conditions. Slices 1 to 3 are shown from top to bottom. Ctrl = control

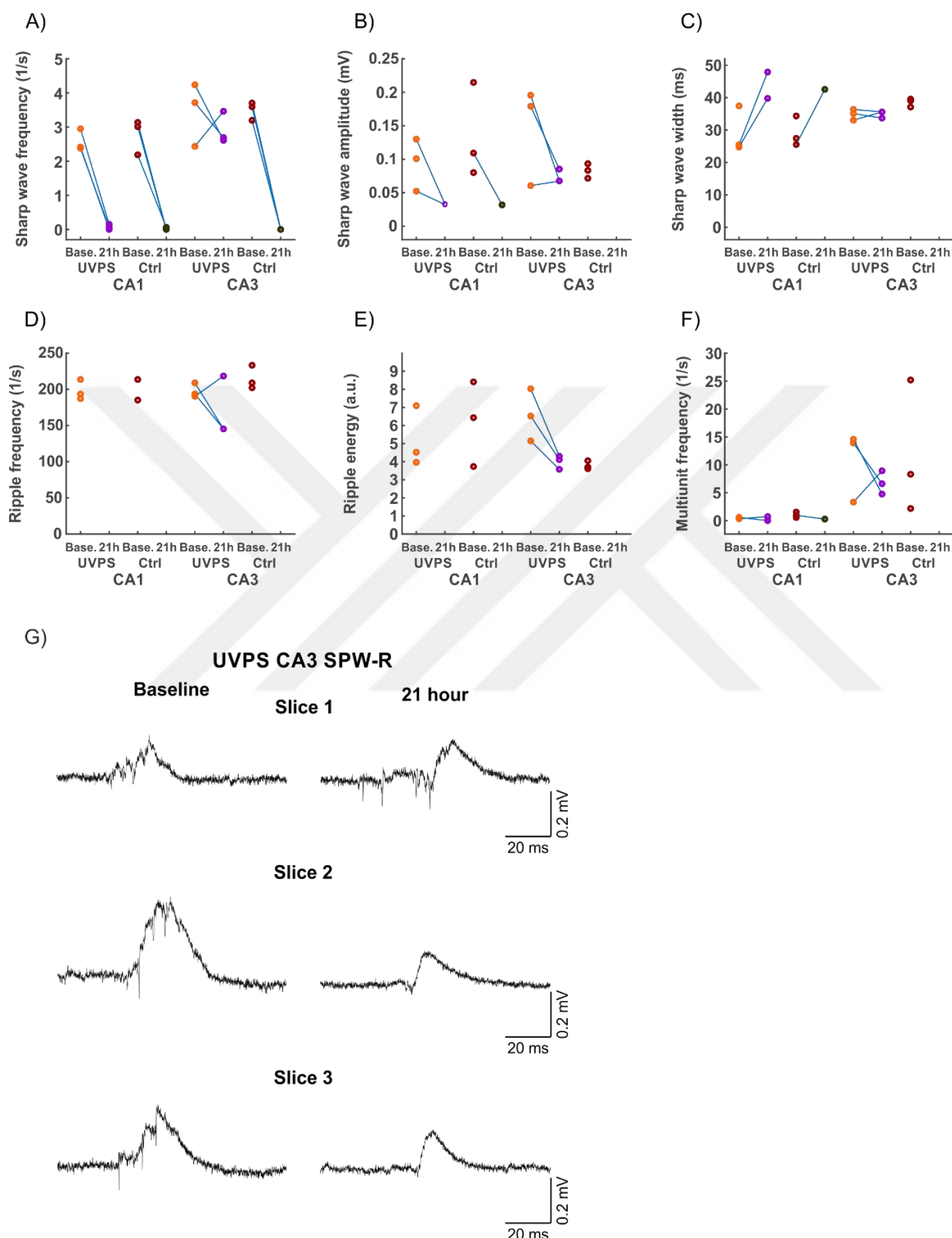


Figure 29: Properties of SPW-R in CA1 and CA3 during baseline and after 21 hours of incubation, comparing UVPS and control slices and 100 millisecond example traces of tetraode recordings taken from CA3 stratum pyramidale of UVPS slices. A-F) Plots comparing the network oscillation properties and multiunit frequency of the CA1 and CA3 regions of UVPS and control slices during baseline and 21-hour conditions. Each value is presented as a dot. In all plots, from left to right the

values shown are; UVPS CA1 Baseline (orange colored), UVPS CA1 21-hour (purple colored), control CA1 Baseline (dark red colored), control CA1 (green colored), UVPS CA3 Baseline (orange colored), UVPS CA3 21-hour (purple colored), control CA3 Baseline (dark red colored), control CA3 (green colored) Because of the low slice number (n=3) statistics such as boxplots and calculation of P values could not be performed. At 21-hour, some slices had no network activity that could be analyzed by the MATLAB algorithm, thus in the plots these values are omitted and there is no dot or fewer dots at the 21-hour time points. **A)** Sharp wave Frequency (1/s). **B)** Sharp wave Amplitude (mV). **C)** Sharp wave Width (ms). **D)** Ripple Frequency (1/s). **E)** Ripple Energy (arbitrary unit, a.u.) **F)** Multiunit Frequency (1/s). **G)** Example traces of 100 milliseconds tetrode recordings showing a single Sharp wave-Ripple Complex (SPW-R) taken from UVPS CA3 during baseline (left column) and 21-hour (right column) conditions. Slices 1 to 3 are shown from top to bottom.

Base. = Baseline, 21h = 21-hours, Ctrl = control

3.2. Histology Results

3.2.1. Mild OGD (OGD until SD) of aged mice (Post-natal week 75 to 104)

AIS properties were not affected significantly at four hours after the onset of SD

It is known that AIS can change their length in response to outside stimuli, a phenomenon called AIS plasticity (Jamann et al., 2018). According to previous findings, AIS can lengthen to increase the excitability of a neuron in response to low levels of excitation and can shorten to decrease the excitability of a neuron in response to high levels of excitation (Jamann et al., 2018). To test this hypothesis, both OGD and control slices were stained for β 4-Spectrin for the deep red channel after being fixated in PFA four hours post SD of the OGD slices. As mentioned before, these cells already emit fluorescent light in the red channel due to expressing tdTomato in their PV + INT. So co-imaging of both the AIS and PV + INT for these slices were possible.

The number of AIS and the median length of AIS of the CA1 and CA3 pyramidal cell layer and the hilus of the DG was measured and the OGD and control slices were compared for significance (see Methods section for a detailed explanation of imaging and measurements). For all regions, the number of AIS and the median AIS length had no significant difference between OGD and control slices (Figure 30, A, B).

The 20x z-stack images used for AIS number and median length measurements didn't have enough resolution to distinguish the AIS of the PV + INT which were inside the pyramidal cell layer of the CA1 and CA3 regions and the hilus region of the DG (Figure 30, D). So the AIS of the PV + INT that were outside of these sub-regions were used for AIS length measurements. Due to low number of PV + INT of each slice, PV + INT of all OGD and control slices were

pooled and the AIS length of these OGD and control cells were compared. There was no significant difference of the PV + INT AIS length in any of the regions (Figure 30, C).

To get images of the CA1 pyramidal cells with greater resolution, 60x images were taken for 8 OGD and 8 control slices and the PV + INT AIS length of these images were measured and again due to low number of cells for each slice, the OGD and control PV + INT were pooled together and compared. There was no significant difference of the PV + INT AIS length of the CA1 pyramidal cell layer between OGD and control slices (Figure 31, A). After the PV + INT were pooled together, the percentage of PV + INT with AIS of OGD and control slices were measured and compared using a Chi-Square ratio test (Figure 31, B). Again there was no significant difference. So, at four hours after SD, the AIS length and number as well as the PV + INT AIS length and percentage of PV + INT with AIS didn't show significant difference in OGD slices compared to control slices, following the mild OGD of acute brain slices of old mice. Example images of PV + ITN with AIS of OGD and control slices are shown in Figure 31, C.

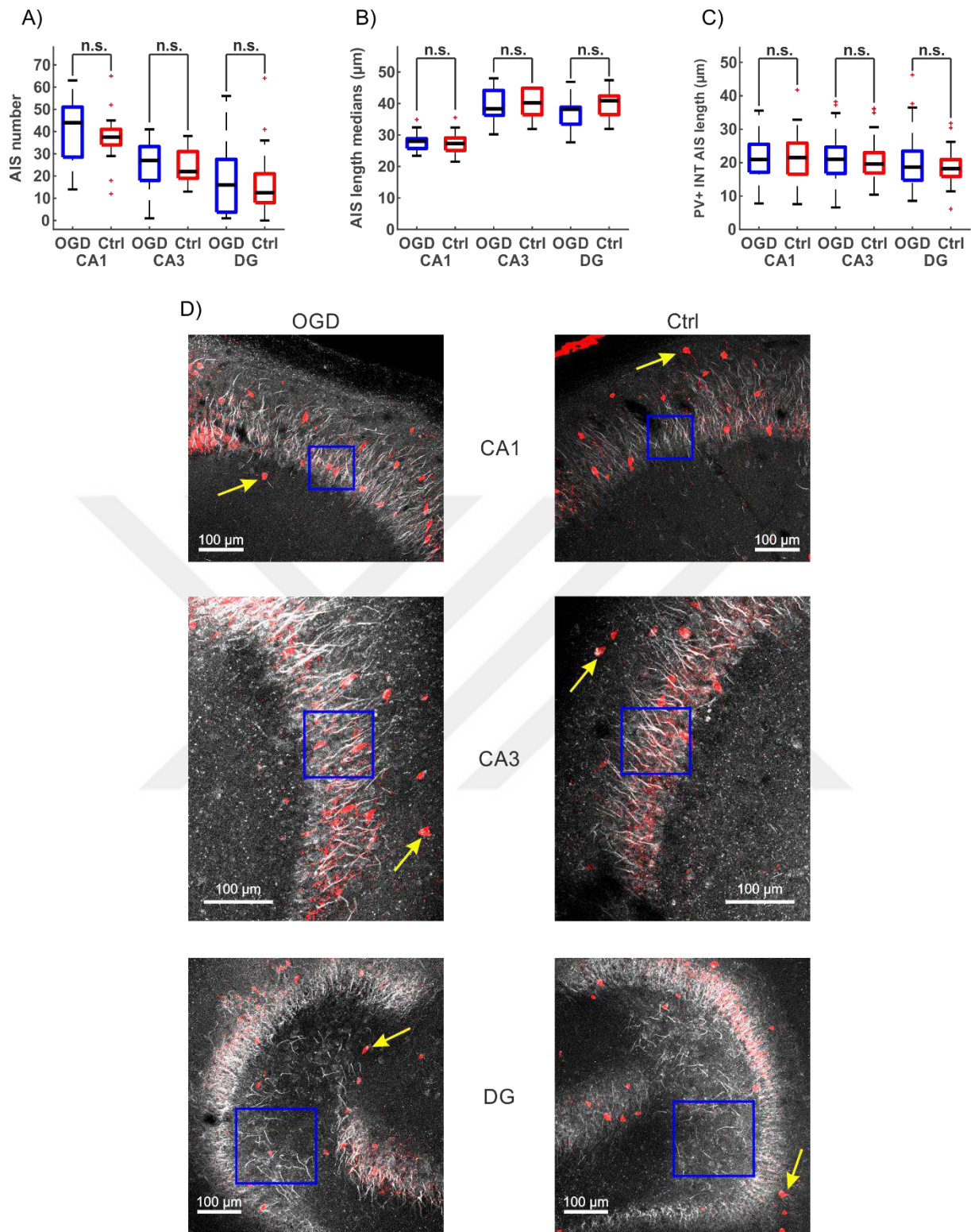


Figure 30: AIS length and number in CA1, CA3 and Dentate Gyrus (DG) comparing OGD and control slices and example images that show the regions of interest for AIS length and median calculations as well as interneurons outside the pyramidal cell layers that were measured for AIS length. A-C) Boxplots comparing the CA1, CA3 and DG regions of OGD and control slices that were fixated in 1 % PFA four hours after the Spreading Depression was recorded in the OGD slices, in all boxplots the regions shown from left to right are; OGD CA1 (blue colored), control CA1 (red colored), OGD CA3 (blue colored), control CA3 (red colored), OGD DG (blue colored), control DG (red colored).

All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. **A)** AIS Number, measured by counting the AIS in a $10011.68 \mu\text{m}^2$ square selected by the experimenter for the CA1 and CA3 pyramidal cell layer and the AIS in a $32444 \mu\text{m}^2$ rectangle selected by the experimenter for the hilus region of the dentate gyrus (DG). $p_{\text{CA1}} = 0.37$, $p_{\text{CA3}} = 0.61$, $p_{\text{DG}} = 0.69$, $n_{\text{OGDCA1}} = 17$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA1}} = 18$ slices taken from the same mice, $n_{\text{OGDCA3}} = 17$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA3}} = 18$ slices taken from the same mice, $n_{\text{OGDDG}} = 17$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLDG}} = 18$ slices taken from the same mice. **B)** AIS length medians, calculated by measuring the length of 10 AIS from CA1 and CA3 pyramidal cell layer and 10 AIS from the hilus region of DG and taking the median value of the AIS lengths. $p_{\text{CA1}} = 0.73$, $p_{\text{CA3}} = 0.90$, $p_{\text{DG}} = 0.07$, $n_{\text{OGDCA1}} = 17$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA1}} = 18$ slices taken from the same mice, $n_{\text{OGDCA3}} = 16$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA3}} = 18$ slices taken from the same mice, $n_{\text{OGDDG}} = 13$ slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLDG}} = 15$ slices taken from the same mice. **C)** AIS length of Parvalbumin positive interneurons (PV + INT), calculated by measuring the length of the AIS from the PV + INT of the CA1 and CA3 regions that are preferably not within the pyramidal cell layer and PV + INT from the general DG region. $p_{\text{CA1}} = 0.81$, $p_{\text{CA3}} = 0.71$, $p_{\text{DG}} = 0.65$, $n_{\text{OGDCA1}} = 36$ PV + INT AIS measured from 12 slices from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA1}} = 50$ PV + INT AIS measured from 17 slices taken from the same mice, $n_{\text{OGDCA3}} = 48$ PV + INT AIS measured from 16 slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLCA3}} = 37$ PV + INT AIS measured from 14 slices taken from the same mice, $n_{\text{OGDDG}} = 38$ PV + INT AIS measured from 13 slices taken from 11 mice aged 75 to 104 weeks, $n_{\text{CTRLDG}} = 38$ PV + INT AIS measured from 12 slices taken from the same mice. **D)** Example images of the stained hippocampal slices. Red colored cells are the PV + INT and the white colored lines are the AIS, (specifically the β -4 spectrin). Left column are OGD and right column are control, from top to bottom, the regions shown are CA1, CA3 and the DG. Blue boxes indicate the approximate sections of these regions where AIS length and number were measured from. Yellow arrows point to exemplary PV + INT that were outside the pyramidal cell layers that were used in AIS length measurements.

n.s. = not significant, p = p value, Ctrl = control

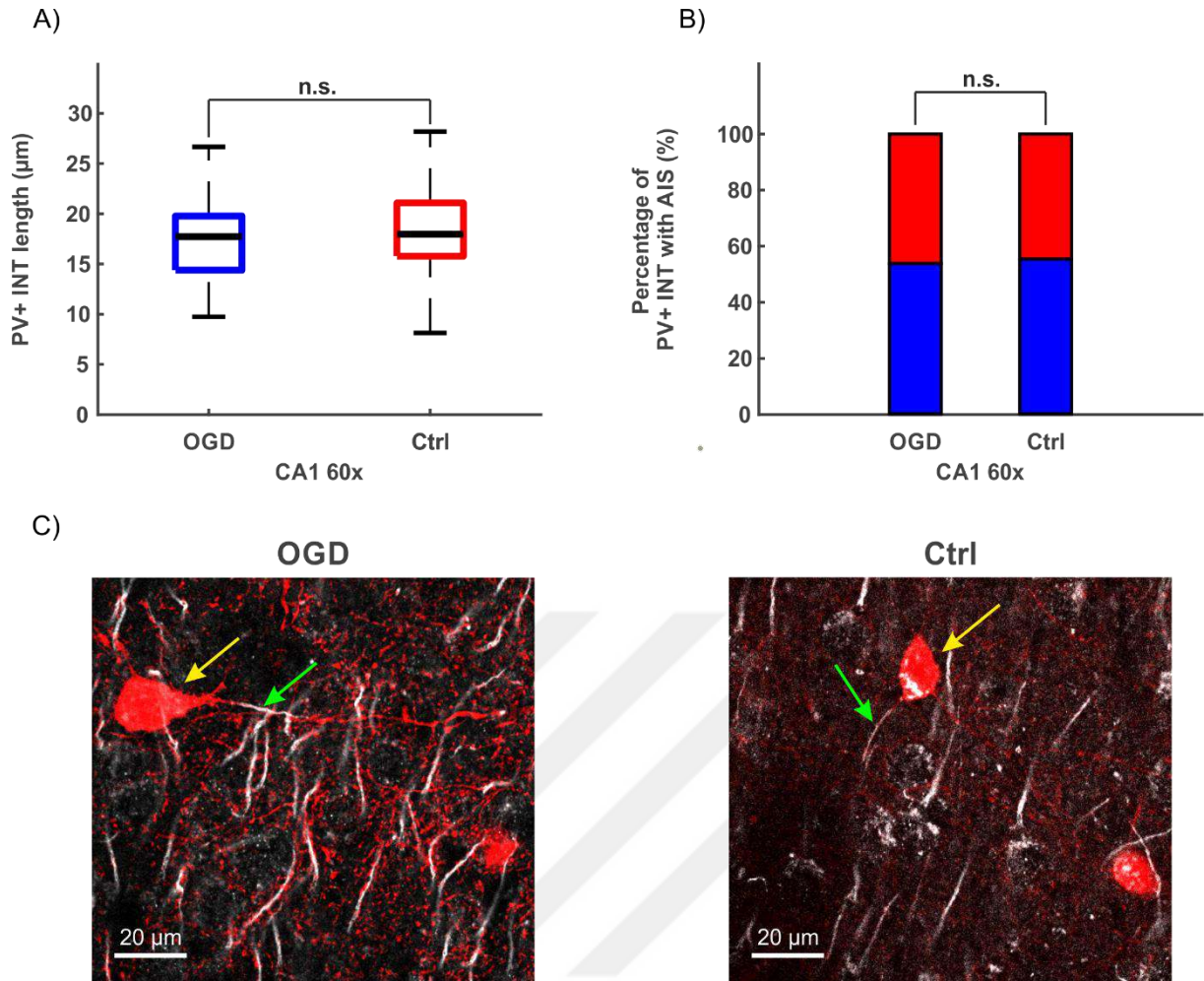


Figure 31: Comparing the length of the PV + INT AIS and comparing the percentage of PV + INT with AIS of the CA1 pyramidal cell layer of OGD and control slices and exemplary images showing PV + INT with AIS in the CA1 pyramidal cell layer. A) PV + INT AIS length boxplots comparing the CA1 pyramidal cell layer magnified at 60x of the OGD (blue colored) and control (red colored) slices. $p = 0.58$, Wilcoxon rank-sum (Mann-Whitney U) test. **B)** Percentage of PV + INT with AIS boxplots comparing the CA1 regions magnified at 60x of the OGD (left) and Ctrl (right) slices. $p = 0.85$, Chi-Square test. $n_{\text{OGDCA1}} = 42$ PV + INT AIS from 8 slices collected from 4 mice aged 75 to 104 weeks (42 out of 78 PV + INT had AIS). $n_{\text{CTRLCA1}} = 36$ PV + INT AIS from 8 slices collected from 4 mice aged 75 to 104 weeks (36 out of 65 PV + INT had AIS). **C)** Example images of the CA1 pyramidal cell layer of OGD (left) and control (right) slices, yellow arrows point to the red colored PV + INT and the green arrows point to the white colored AIS of these PV + INT.

n.s. = not significant $p = p$ value, Ctrl = control

3.2.2. 21-hour UVPS of P60-70 mice

AIS morphology of the CA1 pyramidal cell layer of the UVPS and control slices didn't have a significant difference following 21 hours of incubation

Following the incubation of slices for 21 hours, the UVPS and control slices were fixated in PFA overnight and stained and 60x z-stack images of the CA1 pyramidal cell layer of these slices were taken (see Method section). Further image analysis of the 60x z-stack images of the red channel (parvalbumin) and the deep red channel (β 4-Spectrin) were performed with Fiji Image J (Figure 32, E shows example images of a PV + INT cell with its AIS for UVPS and control slices). The AIS length median (Figure 32, A) and the number of AIS (Figure 32, B) was not significantly different for the CA1 region of the UVPS and control slices, following 21 hour incubation.

Due to low number of PV + INT cells per slice, the PV + INT of the UVPS and control slices were pooled. No significant difference was found in the AIS length of PV+ INT (Figure 32, C) and the percentage of PV + INT with AIS was compared in between UVPS and control slices using a Chi-square ratio test and there was no significant difference between UVPS and control slices (Figure 32, D).

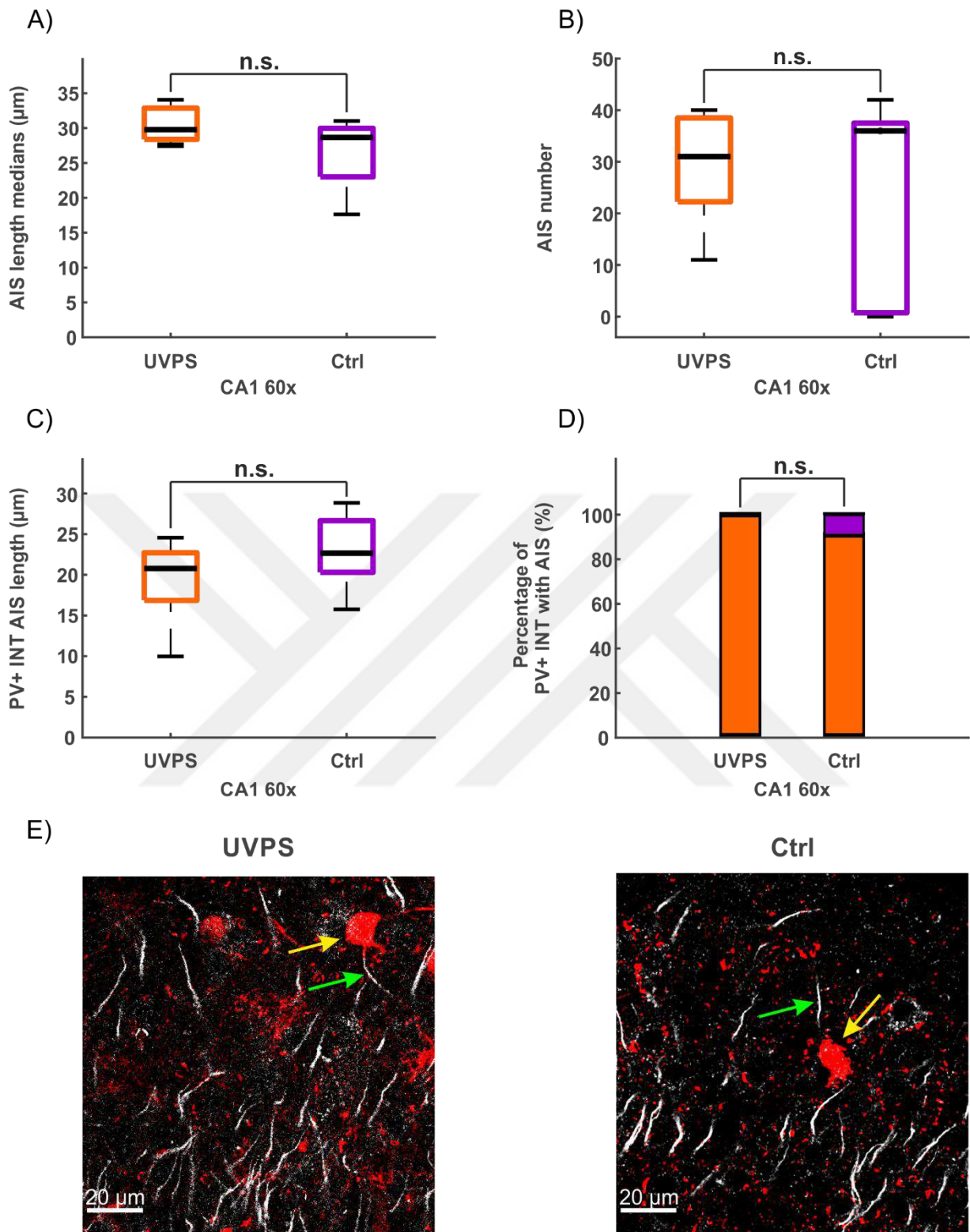


Figure 32: AIS properties of the CA1 pyramidal cell layer, comparing UVPS and control slices and example images that show the CA1 pyramidal cell layer of UVPS and control slices showing the red colored PV + INT and the white colored AIS. A-C) Boxplots of AIS properties comparing the CA1 pyramidal cell layer magnified at 60x of UVPS (orange colored) and control (purple colored) slices. All p values are calculated using the Wilcoxon rank-sum (Mann-Whitney U) test. **A) AIS length medians, calculated by measuring the length of 20 AIS (or how many are available) of each slice and calculating the median of it, $p = 0.41$. **B)** AIS numbers, measured by counting the AIS in a 10051.94**

μm^2 square selected by the experimenter, $p = 0.80$, $n_{\text{UVPSCA1}} = 5$ slices taken from 2 mice aged postnatal day 60-70, $n_{\text{CtrlCA1}} = 4$ slices taken from the same mice. **C)** PV + INT AIS length, $p = 0.14$. **D)** Percentage of PV + INT with AIS, $p = 0.33$, Chi-Square test. $n_{\text{UVPSCA1}} = 10$ PV + INT AIS from 3 slices collected from 2 mice aged postnatal day 60-70, (10 out of 10 PV + INT had AIS), $n_{\text{CtrlCA1}} = 10$ PV + INT AIS from 3 slices collected from the same mice, (10 out of 11 PV + INT had AIS). **E)** Example images of the CA1 pyramidal cell layer of the UVPS (left) and control (right) slices, yellow arrows point to the red colored PV + INT and the green arrow point to the white colored AIS of these PV + INT.

n.s. = not significant, p = p value, Ctrl = control

3.2.3. 20-minute OGD of P28-40 mice

Number of AIS was reduced significantly compared to control slices for the slices fixated after four hour recovery following 20-minute OGD

Following 20-minute OGD, the OGD and control slices were fixated in PFA immediately after or following one hour or four hour recovery and stained for $\beta 4$ -Spectrin to see whether recovery time plays a part on the effect of 20-minute OGD on AIS morphology. Afterwards 60x z-stack images were taken of both 20-minute OGD and control slices and analyzed on Fiji Image J for number of AIS (see Methods). The number of AIS between 20-minute OGD and control slices was not significantly different for the slices that were fixated in PFA immediately after the OGD was performed. However, the number of AIS was reduced almost significantly for 20-minute OGD slices compared to control slices for the slices fixated following one hour recovery ($p = 0.071$) and the number of AIS was reduced significantly for 20-minute OGD slices compared to control slices for the slices fixated following four hour recovery ($p = 0.029$) (Figure 33, A).

To show this effect of reduction of the AIS number better, hippocampus images magnified to 4x of 20-minute OGD and control slices are shown (Figure 33, B). From these images, it can be seen that not only the CA1 region, but the entire CA1, CA2 and CA3 regions have reduced numbers of AIS at one hour and four hour recovery from 20-minute OGD. However, this effect was not quantified or statistically analyzed for the CA2 and CA3 regions. Overall, it is shown that following strong (20 minute) OGD, there is a clear and delayed reduction of the number of AIS in the CA1 pyramidal cell layer which is not manifested immediately after the 20-minute OGD.

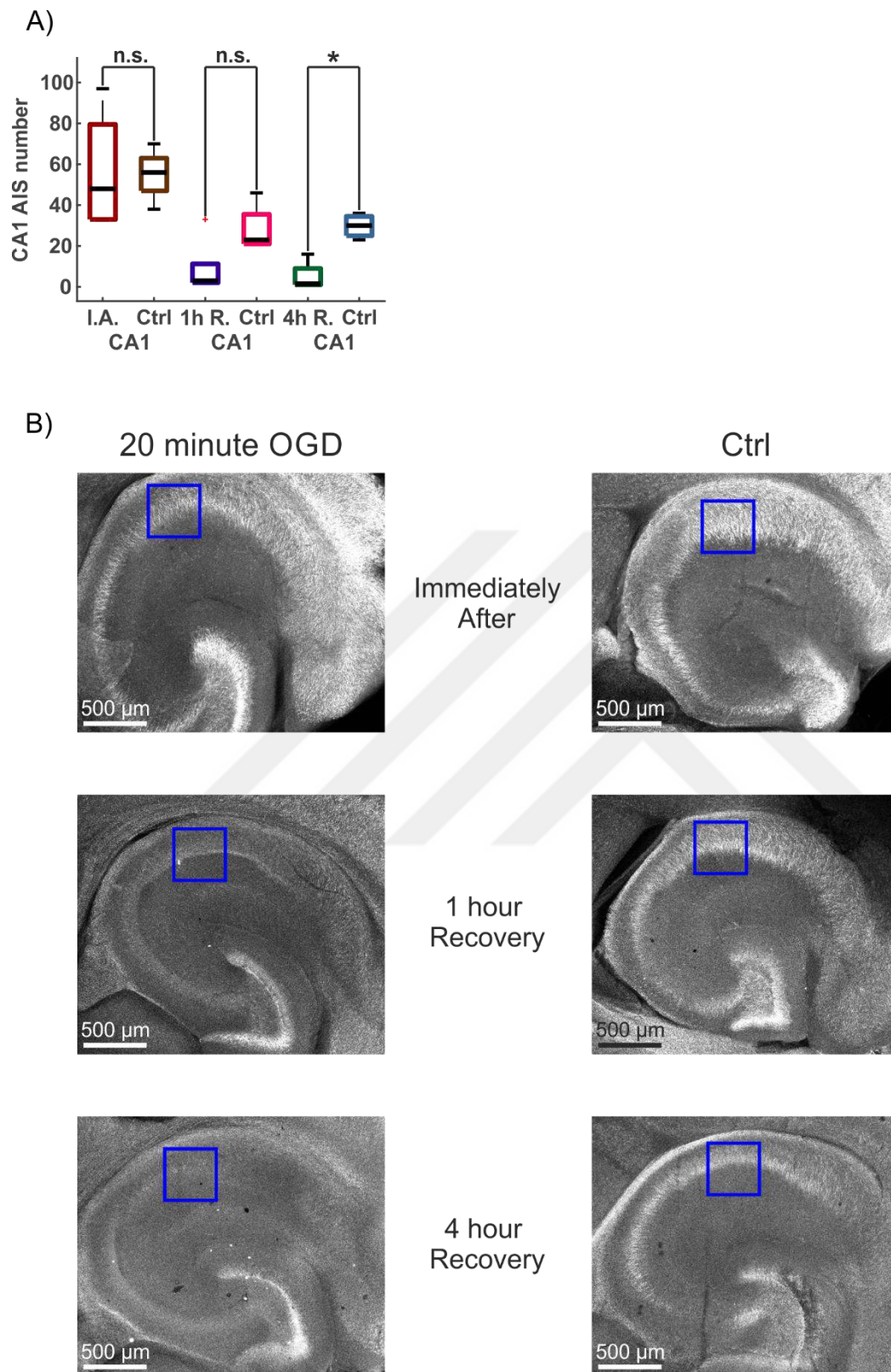


Figure 33: AIS numbers of the CA1 pyramidal cell layer comparing 20-minute OGD and control slices and example images that show the entire hippocampus of 20-minute OGD and control slices with the AIS colored as white. A) AIS Number boxplots of CA1 pyramidal cell layer of 20-minute OGD and control slices that were put into PFA at different time points. The CA1 regions shown from left to right are; 20-minute OGD I.A. (put into PFA immediately after 20-minute OGD), (red colored),

control I.A. (brown colored), 20-minute OGD one hour recovery (put into PFA after one hour recovery from 20-minute OGD), (purple colored), control one hour recovery (pink colored), 20-minute OGD four hour recovery (put into PFA after four hours of recovery from 20-minute OGD), (light green colored), control four hour recovery (light blue colored). AIS numbers, measured by counting the AIS in a $10051.94 \mu\text{m}^2$ rectangle selected by the experimenter. $p_{\text{I.A.}} = 0.86$, $p_{1\text{hR}} = 0.071$, $p_{4\text{hR}} = 0.029$. $n_{\text{I.A.}} =$ four 20-minute OGD and control slice pairs taken from 3 mice aged postnatal day 28-40, $n_{1\text{hR}} =$ five 20-minute OGD and control slice pairs taken from 3 mice aged postnatal day 28-40, $n_{4\text{hR}} =$ four 20-minute OGD and control slice pairs taken from 3 mice aged postnatal day 28-40. **B)** Example images of the entire hippocampus of 20-minute OGD (left) and control (right) slices showing the white colored AIS. From top the bottom; immediately after, one hour recovery and four hour recovery. The lack of AIS can be seen for the one hour and four hour recovery of 20-minute OGD slices. The blue squares indicate the approximate regions where CA1 pyramidal cell layer images at 60x magnification were taken which were used for AIS number measurements.

I.A. = immediately after, 1h R. = one hour recovery, 4h R. = four hour recovery, n.s. = not significant, $* = p < 0.05$, $p = p$ value, Ctrl = control



4. DISCUSSION

In this thesis, three main experiments were performed. Firstly, mild OGD (OGD until SD) was performed on acute hippocampal slices of mice aged 75-104 weeks, which is a novel area of research as no such experiment was performed before. Through tetrode recordings recorded at baseline, one hour post SD and four hours post SD, it was found that the SPW-R frequency was reduced at one hour post SD and four hours post SD compared to baseline for both CA1 and CA3 regions without a significant change in other SPW-R properties such as ripple frequency, ripple energy, sharp wave width and amplitude. The persistent reduction of SPW-R frequency at one hour and four hours post SD shows that mild OGD disrupts SPW-R and this disruption is persistent.

At one hour post SD single unit firing was reduced for both CA1 and CA3 regions. Looking at separated units, PYR firing remained at same level whereas the MIX units with higher baseline frequencies and the 2 FSI units had reduced firing rates, indicating that fast-spiking interneuron firing was affected by mild OGD whereas pyramidal cell firing wasn't. Unit to SPW-R coupling of the CA1 region was also higher compared to baseline, indicating that as unit firing decreased units fired more exclusively during SPW-R since SPW-R provides strong excitation (Both et al., 2008, Buzsáki, 2015). At four hours post SD, the PYR firing rate of both CA1 and CA3 region were significantly higher for mild OGD slices compared to control slices and the FSI firing rate of mild OGD slices were almost significantly lower ($p=0.052$) compared to control slices. Indicating that as FSI firing became disrupted following mild OGD, pyramidal cell firing increased due to disinhibition and units became less coupled to SPW-R, which disrupts the temporal coding of the hippocampal network since unit coupling to SPW-R is an important aspect of the information processing of the hippocampal circuit (Buzsáki, 2015).

Following 21-hour incubation, compared to control slices that showed no SPW-R activity in CA1 and CA3 regions, hippocampal slices from P60-70 mice placed in the UVPS set-up were able to maintain SPW-R in their CA3 regions even though the SPW-R had decreased amplitude and decreased ripple energy and frequency compared to baseline. However, the CA1 region of these UVPS slices also showed no SPW-R activity, possibly due to CA1 being selectively vulnerable to metabolic and cytotoxic stress (Bartsch et al., 2015). Another reason might be the reduced quality of the CA3 SPW-R after 21-hour incubation leading to them not being relayed to the CA1 region, since it is known that SPW-R is relayed to the CA1 region after initiating in the CA3 region (Both et al., 2008, Schlingloff et al., 2014).

The AIS length and number of PYR and PV+ INT of mild OGD slices did not show any significant difference from control slices, possibly because the change in frequency at four hours post SD for pyramidal cells and fast-spiking interneurons are mild and four hour recovery time is not enough for AIS length to alter. Another reason could be that following mild OGD FSI are suffering from dysfunctions that also alter their ability to adapt their AIS to changes in excitation which happens under normal conditions (Jamann et al., 2018). Similarly, the AIS length and number of PYR and PV + INT of the CA1 region of UVPS slices were not altered compared to control slices, which can be due to the low number of these slices (4 UVPS and 5 control slices), or it could be because the CA1 neurons of both UVPS and control slices went through apoptosis and ceased to show AIS plasticity.

Following 20-minute OGD, the majority of AIS in the CA1 region seemed to remain intact compared to control slices, if slices were fixated in 1 % PFA immediately after the insult. However, for slices that were fixated one hour and four hours after the insult, there were significantly less AIS in the CA1 region compared to control slices which could be a result of cell death, or AIS proteolysis which is known to be a sign of neuronal injury that can occur without cell death or axonal degeneration (Schafer et al., 2009).

4.1. Effect of mild OGD on SPW-R

Immediately following mild OGD, the SPW-R oscillation was non-existent for some time in both CA1 and CA3 regions, but as time went on, there was a gradual recovery of SPW-R even though the frequency of sharp waves remained lower than baseline for both regions (Figure 15 G, H). At one hour post SD, the sharp wave frequency was significantly reduced for both CA1 and CA3 regions, without any other significant change observed for sharp wave properties such as ripple frequency, ripple energy etc. Comparing the normalized SPW-R frequency values of mild OGD and control slices at four hours post SD also showed that mild OGD slices had significantly lower normalized frequency for the CA3 region compared to control slices and the SPW-R frequency of the CA1 region of mild OGD slices was almost significantly lower ($p=0.073$). This p value could be due to the relatively low number of slices for this experiment (10 mild OGD slices and 11 control slices for the CA1 region). At four hours post SD, there wasn't any significant difference between mild OGD and control slices for other SPW-R properties except for sharp wave width, which was significantly larger for the CA3 region of the control slices compared to mild OGD slices.

Interestingly, it also seems that the reduction of the SPW-R frequency compared to baseline enhanced as time went on. At one hour post SD, the CA1 SPW-R frequency went from 2.88 Hz [2.69 3.076], to 2.16 Hz [0.74 2.76], so there was a 25 % reduction (Figure 15, E). At four hour post SD, the normalized CA1 SPW-R frequency of the mild OGD slices was 0.39, so there was a 61 % reduction (Figure 18, E). For the CA3 region, following the same calculations, at one hour post SD there was a 33 % reduction of SPW-R frequency (Figure 15, E) and at four hours post SD, there was a 46 % reduction (Figure 18, E).

It is reasonable for the SPW-R to cease during mild OGD and only recover partially four hours post SD since it is known that network oscillations are high-energy consuming processes (Kann et al., 2011) and they would be the most affected and be the first thing that is disrupted by metabolic stress. If there is a lasting effect on the energy consumption of neurons after mild OGD, even if the effect is small, it would be expected to disrupt SPW-R. It is also known that it takes some time for the damage of OGD to neurons to manifest such as apoptosis (Mattson et al., 2000, Lai et al., 2014). As time goes on and more neurons go through apoptosis, it is expected that the mild OGD slices would have an increased difficulty in maintaining SPW-R leading to increased reduction of frequency as time goes on.

Another possible explanation of how mild OGD leads to a reduction in SPW-R frequency that enhances as time goes on, could be speculated by looking at the unit activity of these slices. As seen in the results section, pyramidal cells of the mild OGD slices had significantly larger frequency than the control slices at four hours post SD for both CA1 and CA3 regions, and the fast-spiking interneurons of the mild OGD slices had lower frequency than the control slices which was almost significant ($p=0.052$). This suggests that the inhibition excitation balance of the network is disrupted for the mild OGD slices and it is known that a precondition of SPW-R generation is a high inhibition/excitation (I/E) ratio (Hájos et al., 2013; Schlinghoff et al., 2014). Thus, a decrease in interneuron firing and an increase in pyramidal cell firing could result in a reduction in the overall occurrence of SPW-R which was observed, since it would be more difficult and less frequent for the neurons to meet the high I/E ratio precondition. If this experiment is repeated with a longer recovery period, it could be seen that as time goes on, if the inhibitory neuron firing decreases and the pyramidal neuron firing increases more and more, it would be less and less likely for the SPW-R precondition of high I/E ratio to be met and the SPW-R frequency would continue to decrease to the point of cessation.

Another possible reason that can't be dismissed for the gradually increasing reduction of SPW-R frequency of mild OGD slices with time, is the possibility that as the slices remain in the

interface chamber they gradually decline in health due to bacterial growth. (Buskila et al., 2014). Therefore, the initial reduction in SPW-R frequency might be compounded due to increased time the slices spend in the interface chamber and not directly be caused by mild OGD. For comparison, at four hours post SD, SPW-R frequency of the CA1 region of the control slices was reduced 31 % compared to baseline and SPW-R frequency of the CA3 region was reduced 4 % compared to baseline (Figure 18, E). These values are smaller than the SPW-R frequency reduction of mild OGD slices at four hours post SD, but there was still a reduction, showing that the slices seem to be affected by just being in the interface chamber. The larger reduction of the CA1 SPW-R frequency in both OGD and control slices compared to the CA3 region at four hours post SD is consistent with the findings that suggest CA1 region is selectively vulnerable to metabolic stress (Bartsch et al., 2015).

To see whether the enhancement of the reduction of SPW-R frequency as time goes on is caused by the mild OGD, more recordings can be taken at different time points, at two, three and four hours post SD, or even better for longer recovery periods such as six hours or eight hours post SD. If the rate of the reduction of the SPW-R frequency is higher for the mild OGD slices in between each time point than the rate of reduction for the control slices, than the most probable cause of this phenomenon would be mild OGD. It would also be valuable to conduct experiments with young and old mice combined to see whether the persistent reduction in frequency of SPW-R at four hours post SD would also be observed for young mice following mild OGD.

4.2. Effect of mild OGD on multi-unit and single unit activity

Following mild OGD, multi-unit activity (MUA) ceased for a short time as it is expected since neurons are deprived of energy but in accordance with previous findings, following re-oxygenation, neurons regained their ability to fire spontaneously (Hefter et al., 2016). MUA frequency was significantly reduced for the CA1 region at one hour post SD and almost significantly reduced for the CA3 region ($p=0.064$) and the unit frequency of CA1 and CA3 regions were also significantly reduced. This reduction of frequency of unit firing is in line with previous findings (Grube et al., manuscript in print).

The MUA and unit to SPW-R coupling (sharp wave specificity) of the CA1 region significantly increased one hour post SD compared to baseline. Together with the finding of the reduction of unit frequency, this finding suggests that for the CA1 region, as the unit frequency is reduced at one hour post SD, only units which are strongly coupled to SPW-R oscillation are able to

fire. Another explanation could be that as unit firing decreases at the CA1 region, units tend to fire proportionally more during SPW-R's since SPW-R's are known to stimulate unit firing (Both et al., 2008). There was also a significant decrease in the degree of the median ripple phase where MUA occurred as well as a significant decrease in the ripple phase specificity of the CA1 region at one hour post SD, meaning MUA became less coupled to phases of the ripple oscillation. This could also result in a disruption to information coding of the hippocampal network.

After the units were separated into PYR, MIX and FSI units, it was found that at one hour post SD, the frequency of the two FSI units were reduced as well as MIX units of both CA1 and CA3 regions. Contrary to this, PYR units showed no significant reduction of frequency in both CA1 and CA3 regions at one hour post SD. Looking more in detail to MIX units boxplots, it was observed that MIX units with higher baseline frequency showed a decrease in frequency while MIX units with smaller baseline frequency didn't show a decrease, implying that interneurons with high baseline frequency seemed to be affected more by mild OGD than pyramidal cells at one hour post SD. The unit to SPW-R coupling (sharp wave specificity) of MIX units of the CA1 region was almost significantly decreased at one hour post SD ($p=0.055$), reinforcing the idea that for the CA1 region, only units strongly coupled to SPW-R are able to fire at one hour post SD or that as unit frequency decreases, units fire proportionally more during SPW-R's.

At four hours post SD, the unit firing rate of the CA3 region of mild OGD slices was higher than control slices. The unit firing rate of the CA1 region was also higher for mild OGD slices, although the difference was not significant. This finding was unexpected since a previous finding showed decrease of unit frequency at one hour post SD (Grube et al., manuscript in print), however, four hours post SD recordings were not taken for this paper. The sharp wave specificity of the units of the CA1 region were significantly smaller for mild OGD slices compared to control slices, which indicated that the units of the CA1 region became less coupled to SPW-R at four hours post SD, which would increase the risk of cells firing with less precision and disrupt the information coding of the CA1 neuronal network since it is achieved by a strong coupling of unit firing to SPW-R (Buzsáki, 2015).

Looking at separated units, the PYR frequency of the mild OGD slices were significantly higher compared to control slices for both CA1 and CA3 regions, while the FSI frequency of OGD 4h slices were almost significantly lower compared to control slices ($p=0.052$) which could have been significant if more than 5 mild OGD and 6 control interneurons were detected. The sharp

wave specificity of MIX units of OGD 4h slices was also significantly lower compared to control slices again showing that CA1 unit to SPW-R coupling decreased at four hours post SD which shows that the risk of a disruption to information coding of the CA1 network continues at four hours post SD.

The increased unit frequency of the CA3 region at four hours post SD for mild OGD slices were deemed as unexpected since at one hour post SD, unit frequency was lowered. However, looking at the result of increased PYR unit frequency and decreased FSI unit frequency at four hours post SD it could be said that PYR cells fire more frequently due to disinhibition. This is in line with a previous finding that showed an increase in pyramidal cell firing possibly due to disinhibition following mild metabolic stress caused by the injection of rotenone (Elzoheiry et al., 2020). At one hour post SD, there was an implication that FSI firing rate was reduced but PYR firing rate didn't show an increase as they did at four hours post SD. This could be due to the overall depression of unit activity caused by mild OGD so it could be that it takes some time for the PYR units to return to baseline frequency and exceed baseline frequency.

There were no significant changes of any type of unit's coupling to SPW-R for the CA3 region, unlike the CA1 region. Looking at baseline multi-unit and single unit sharp wave specificity boxplots, (Figure 12, B and Figure 13, B), it can be seen that at baseline CA3 units were less coupled to SPW-R than CA1 units, and CA1 units showed almost complete coupling to SPW-R. Perhaps this means that since for the CA3 units don't show as much coupling to SPW-R at baseline, small changes in unit firing rate don't translate to changes in SPW-R coupling for the CA3 region as well as it seems to for the CA1 region.

Interneuron firing, especially PV + FSI, is important in maintaining the ripple oscillations since these neurons form recurrent networks that upon activation leads to a fast oscillation (150-200 Hz) (Schlingloff et al., 2014), so the decrease in interneuron firing at one hour and four hours post SD could have resulted in a reduction of the ripple frequency. However, this was not observed, which could mean that the disruption of FSI firing was not large enough to effect ripple oscillations even though it affected PYR firing. It could also be that it takes longer for the disruption in FSI firing to start affecting ripples so if there was a longer recovery period the reduction in ripple frequency may have been observed.

4.3. Effect of the 21-hour UVPS method on SPW-R

In order to increase the recovery time of slices from mild OGD, it is necessary to prolong the viability of acute brain slices and as explained more in detail in the methods section, a modified

Haas-type interface chamber was made with the help of a pump and UV light and the hippocampal brain slices of P60-70 mice were incubated in this chamber that used ACSF with low concentrations of penicillin and streptomycin. Control slices were incubated in a regular Haas-type interface chamber without an UV lamp bottle and were incubated with ACSF that didn't contain any antibiotics (See Methods for a detailed explanation). During baseline (2 hours incubation), the SPW-R properties and the MUA frequency of both CA1 and CA3 regions of the 3 brain slices had similar values for UVPS and control slices. This shows that the low concentration of antibiotics used in the UVPS method does not increase the activity of neurons and lead to epileptic episodes even though it is known that penicillin induces epilepsy (Grøndahl and Langmoen, 1993).

Following 21-hours of incubation, it was seen that only the CA3 region of the UVPS slices had SPW-R oscillations. CA1 region of the UVPS slices didn't show any significant SPW-R although some unit firing was seen. This indicates that even though some neurons might function normally, SPW-R oscillation is very fragile and slices have to remain in a healthy condition with most neurons intact to show SPW-R. The CA3 region of UVPS slices had reduced SPW-R amplitude and reduced ripple energy and two of the three UVPS slices had reduced ripple frequency following 21-hours of incubation in the UV set up. This indicates that even though SPW-R is present, they are disrupted for the CA3 region. Reduced ripple energy and frequency of SPW-R might mean FSI are impacted heavily by the 21-hour incubation in the UVPS set up. Reduced SPW-R amplitude also indicates that a smaller number of cells might be participating in the network oscillation. Still UVPS method seems to be superior to control since in the control slices only some unit activity was observed with no SPW-R in both CA1 and CA3 regions.

The CA1 region of the UVPS slices showed no significant SPW-R which could be because the intrinsic selective vulnerability of CA1 region to metabolic as well as cytotoxic stress (Bartsch et al., 2015). In the mild OGD experiment, at four hours post SD, CA1 SPW-R frequency was reduced much strongly than the SPW-R frequency of the CA3 region for both mild OGD and control slices, so this also shows the increased vulnerability of the CA1 region. Another reason for the silencing of the SPW-R of the CA1 region while CA3 region of UVPS slices still had SPW-R could be that as the SPW-R amplitude and ripple energy was disrupted for the CA3 region, the relaying of the SPW-R oscillation onto the CA1 region was also disrupted. This is expected since as mentioned in the introduction, it is known that SPW-R's originate in the CA3 region and are relayed on to the CA1 region (Both et al., 2008, Schlingloff et al., 2014). Thus,

a decrease in the quality of CA3 SPW-R might also weaken the signal of excitation below a certain threshold so that CA1 neurons can't initiate SPW-R. It could also be that Schaffer collaterals which relay information from CA3 to CA1 (Andersen et al., 2007), are especially vulnerable to increased incubation time.

The number of slices (3) was too low for this experiment for any meaningful statistical analysis to be performed. Still, the three UVPS slices that had SPW-R in the CA3 region after 21 hours of incubation show the potential of this method. Future experiments should be performed with increased number of slices and possibly with adjustments to the antibiotic type or concentration until CA1 SPW-R is also observed after overnight incubation. It is known that the antibiotics cefuroxime, clindamycin, cefotaxime, vancomycin, and tobramycin don't lead to epileptic activity when administered to acute rat hippocampal slices (Grøndahl and Langmoen, 1993). These can be used in higher concentrations to test whether they yield better results.

Another possible improvement could be to eliminate the use of gravity in the transfer of ACSF from the one-liter flask to the interface chamber and then to the collection flask (see Figure 7) with the use of pumps. This would maintain a stable ACSF flow throughout the night when the researcher can't intervene if flooding or drying up of the chamber occurs. If slices can be kept healthy to the point that both CA1 and CA3 regions have relatively normal SPW-R after overnight incubation, this would increase the usefulness of the Haas-type interface chambers greatly and mild OGD experiments, or any other experiment, can be performed with increased recovery times. This would lead to more insight regarding the long-term recovery of neuronal networks and individual cells from mild or strong metabolic or cytotoxic stress.

4.4. Effect of mild OGD, 21-hour UVPS and 20-minute OGD on AIS length and number

Mild OGD

After analyzing the mild OGD and control slices which were stained for β 4-Spectrin, it was found that there is no significant difference in the median length and number of AIS of the pyramidal cells of the CA1 and CA3 pyramidal cell layer and the hilus region of the DG. The mice used in this experiment expressed tdTomato fluorescence in their PV + interneurons so it was possible to analyze the PV+ interneuron AIS length. The length of AIS of the PV+ interneurons of mild OGD slices were not significantly different than the control slices. CA1 pyramidal cell layer images were taken at 60x magnification of eight experiment and control slice pairs to see with better resolution the PV+ interneurons of this region and again there was no significant difference between the AIS length of the PV+ interneurons. There was also no

significant difference in the percentage of PV + interneurons with AIS between mild OGD and control slices.

It is known that AIS exhibits plasticity and lengthens in case of lower than usual excitation levels and shortens in case of higher than usual excitation levels (Jamann et al., 2018). At four hours post SD, the pyramidal cells of the mild OGD slices fired with a significantly higher frequency than the control slices for both CA1 and CA3 regions and the fast-spiking interneuron of mild OGD slices fired with an almost significantly lower frequency than control slices. This would lead one to expect the pyramidal cells of the mild OGD slices to have shorter AIS compared to control slices due to increased firing and the PV+ interneurons of mild OGD slices to have longer AIS compared to control due to decreased firing. However, this was not observed. A possible explanation could be that four hour recovery was not enough to see a significant change and that this imbalance of firing for pyramidal cells and interneurons could eventually result in a significant change of the AIS length compared to control slices if the recovery period is longer, such as 8, 16 or even 24 hours. So at four hours, even though excitation levels of neurons are different than normal, the AIS didn't have enough time to respond to this shift in excitation.

Fast-spiking interneurons are known to be especially vulnerable to metabolic stress (Povysheva et al., 2019, Elzoheiry et al., 2020), so these neurons could have a defect in their firing abilities following mild OGD and this could be reason why they exhibit reduced firing rates. If this is the case than these interneurons may never show AIS plasticity because they are not in a healthy state and the problem they have is not a lower than usual excitation level but firing itself. Looking at unit firing results, fast-spiking interneurons are probably still receiving normal levels of excitation or even higher than usual because pyramidal cell firing increases compared to control slices at four hours post SD. So it could be that even as time goes on, these PV+ interneurons will either show no change in AIS because there is disruption in their function or they will show a shortening of AIS due to increased excitation coming from pyramidal cells even though they keep firing at a lower than usual rate. Of course this hypothesis assumes intact pyramidal cell-interneuron synapses that relay the excitation from pyramidal cells with the same quality as before the induction of mild OGD.

21-hour UVPS

There was no significant difference in the AIS length and number of the pyramidal cells of the CA1 pyramidal cell layer between UVPS and control slices. The AIS length of the PV+ interneurons also showed no significant difference as well as the percentage of PV+ cells with AIS. This is again unexpected since looking at the occurrence of SPW-R in the CA3 region of the UVPS slices, it is clear that these slices were more viable than control slices and it would have been an expected result if the control slices had longer AIS due to lower excitation compared to UVPS slices.

There could be several reasons why this was not observed. The most obvious reason is the low number of slices (five UVPS and four control slices) which makes the statistical analysis weak. This experiment should be repeated with more slices to get a better picture of the effect of the 21-hour UVPS method on the AIS. Another reason could be that since the viability of acute brain slices is known to be 6-12 hours in normal conditions and since CA1 region is known to be especially vulnerable to stress (Bartsch et al., 2015), the majority of the CA1 neurons of both UVPS and control slices may have undergone apoptosis after 21 hours of incubation. Once a cell has undergone apoptosis, naturally it wouldn't show any AIS plasticity. The CA3 region of these slices were not analyzed and they could have yielded more information about the AIS since, due to presence of SPW-R in 3 UVPS slices, it is clear for these slices that the CA3 region had more unit activity than the control slices.

20-minute OGD

Following 20-minute OGD of adolescent mice (P28-40) acute hippocampal slices, the experiment and control slice pairs were fixated in 1 % PFA either immediately after 20-minute OGD, or following one hour and four hour recovery. It was found that the AIS number of 20-minute OGD slices were similar to control slices for the immediately after group, but the AIS number of OGD slices were almost significantly lower ($p = 0.071$) for the one hour recovery group and significantly lowered for the four hour recovery group. These findings are surprising since here we see an almost total disappearance of the AIS rather than a change in length. Unfortunately the AIS result of these slices is not accompanied by tetrode recordings so we don't know the unit firing rates of these slices. Of course to get a better idea, this experiment should be repeated with increased number of slices to see whether the disappearance of AIS is persistent and tetrode recordings should be taken to see the change in unit firing and the SPW-R oscillation.

The effect of 20-minute OGD on the cell and network activity would be stronger than mild OGD since 20-minute OGD would put the cells in a more extreme metabolic stress. According to these results, it can be said that 20-minute OGD seems to invoke a response from neurons that leads to total breakdown of β 4-spectrin and hence the AIS structure. Also, this evoked response seems to take some time to manifest, hence the relatively similar number of AIS between 20-minute OGD and control slices that were fixated immediately after the insult. Future experiments should be performed that also stain the cells with DAPI to look at the ratio of pyknotic nuclei or to stain the cells with a biomarker for cell death such as propidium iodide (PI) to see whether this disappearance of AIS is linked to cell death.

4.5. AIS proteolysis in response to neuronal injury

It was shown previously that AIS proteolysis can be an early marker of neuronal injury and it can happen in absence of cell death or axon degeneration (Schafer et al., 2009). Following ischemic injury of up to 90 minutes, achieved by cerebral artery occlusion of mice, there was a significant reduction in β 4-Spectrin immunoreactivity for the neurons that underwent the ischemic insult (Schafer et al., 2009). Researchers also exposed *in vitro* cortical neuron cultures to OGD for up to 3 hours and at 2 hours, AIS of neurons disappeared and PI staining lead to the conclusion that cell death did not occur for neurons without AIS (Schafer et al., 2009). Axonal staining and morphological analysis of stained axons also showed that axons were intact. In view of this finding, it can be said that the total disappearance of AIS of slices that underwent 20-minute OGD can be due to AIS proteolysis without cell death. However, staining cells with PI in future experiments is necessary to come to a definite conclusion.

It was also found that AIS proteolysis occurred under elevated intracellular Ca^{2+} concentrations (Schafer et al., 2009), which is known to be a result of SD (Somjen, 2001). Moreover it was found that submitting the AIS to high levels of Ca^{2+} alone caused AIS proteolysis (Schafer et al., 2009). So it could be that mild OGD doesn't achieve the levels of intracellular Ca^{2+} sufficient to initiate AIS proteolysis but 20-minute OGD, which could possibly yield more than one SD event, does achieve these levels. It is hypothesized that the proteolysis of AIS which leads to a loss of neuronal polarity could be a neuroprotective adaptation in the sense that through rapid loss of AIS and the many sodium channels associated with AIS, the excitability the nerve cells are greatly reduced so they are protected from excitotoxicity which occurs after ischemic stroke (Schafer et al., 2009, Lai et al., 2014). So it could be that neurons that lack AIS are protected from cell death and hence don't show cell death. Blocking of sodium channels

were found to be neuroprotective against ischemic spinal cord injury which also supports this theory (Schwartz and Fehlings, 2001).

Taking the findings about AIS proteolysis into account, again it can be said that mild OGD does not provoke this response whereas 20-minute OGD seems to achieve this. However, the mild OGD and 20-minute OGD experiments performed in this thesis were not performed on animals of the same age and strain. To get a better idea, further experiments should be made using animals of same age and strain with OGD insults of various durations to see the AIS response. Same duration of OGD, preferably 20 minutes or longer to evoke AIS proteolysis, can also be performed on differently aged mice (old and young) of the same strain to see whether AIS proteolysis response changes with age. Achieving longer recovery periods would also be valuable to see the long-term effects of strong OGD on AIS morphology.

4.6. Limitations of study

One of the main limitations of this thesis was the relatively low number of mice and acute hippocampal slices used throughout the various experiments. Most mice were used for the mild OGD experiment which was eleven and for other experiments even less numbers of mice were used and this limits the statistical analysis. Ideally these experiments should be repeated with more number of slices obtained from more mice so that a more reliable statistical result can be obtained.

OGD is a common model employed to simulate hypoxia-ischemia, however, there are some aspects of the model that limits completely accurate simulation. For example, restriction of blood flow is an important component of in-vivo hypoxia-ischemia (Schmidt-Kastner and Freund, 1991) and since acute brain slices lack blood flow altogether, OGD can't be modeled with complete accuracy. Also, before OGD is induced, acute brain slices are perfused with a gas mixture containing 95 % oxygen to keep the slices as healthy as possible and this amount of oxygen is not the natural oxygen levels of the in-vivo brain tissue, so the baseline properties of neuronal activity might be altered in acute brain slices.

As it was written above, the time of viability for acute brain slices is 6-12 hours (Buskila et al., 2014). Considering three hours of incubation pre-OGD, our mild OGD slices were inside the interface chamber for at least seven and a half hours before they were put into PFA. Therefore any possible disruption of the neuronal activity and of the SPW-R can also partially be due to decreased health of the slices as they remain in the chamber for a prolonged amount of time.

The control group partially solves this problem but still in-vivo mid-term effects of mild OGD might be less severe than they are for acute brain slices.

Lastly, even though the spike sorting and clustering algorithm used to detect units is reliable, still the results of this algorithm can't be expected to be 100 % accurate. Some spikes that were sorted into one unit might be from two different units, or vice-versa and some units might be too contaminated with background noise. Part of the reason why these are called 'units' and not 'cells' is that even if the algorithm is pretty advance it can't be said that each unit represent one neuron that was recorded with absolute certainty. Following this, the PYR and FSI units that were determined might also have some degree of uncertainty and some of them might not belong to their assigned groups.

4.7. Conclusion and Outlook

In this thesis, three experiments were performed, mild OGD, 20-minute OGD and 21-hour UVPS incubation, in service of one common goal, to understand the effect of OGD on neuronal network-, and single cell activity and on AIS morphology as well as to find a method that increases the viability of acute brain slices so the long- term effects of mild and strong OGD can be observed.

The mild OGD of old mice (aged 75 to 104 weeks) was a novel experiment as well and it lead to the finding that SPW-R frequency is reduced at one hour post SD for both CA1 and CA3 regions, which is in line with previous findings (Grube et al., manuscript in print), and this reduction in frequency was enhanced at four hours post SD again in both regions. This could be due to a disruption in the inhibition/excitation balance. At one hour post SD, fast-spiking interneurons had reduced firing rate, but pyramidal cells seemed to have a similar firing rate compared to baseline. This contrasts a previous finding that showed that at one hour post SD, pyramidal cell firing was also decreased (Grube et al., manuscript in print).

At four hours post SD, fast-spiking interneurons had reduced firing rates compared to control, and pyramidal cells had increased firing rates which is probably due to disinhibition. This is aligned with a previous experiment that showed increased firing rate of pyramidal cells due to mild metabolic stress caused by rotenone (Elzoheiry et al., 2020). The unit firing of the CA1 region was less coupled to SPW-R compared to control at four hours post SD and this could lead to a disruption of the temporal coding of the hippocampal circuit due to units firing with less precision. (Buzsáki, 2015).

The three slices that were incubated for 21-hours in the UVPS set-up had SPW-R activity in their CA3 region unlike the three control slices, even though SPW-R amplitude, ripple energy and ripple frequency were reduced. CA1 region for both UVPS and control slice showed no SPW-R activity. Possibly due to CA1 region being more vulnerable to metabolic stress or due to CA3 SPW-R not being relayed to CA1 after 21 hours of incubation since CA3 SPW-R was also disrupted to some extent. More experiments should be made with more slices to come to a better conclusion. The antibiotics used as well as their concentrations can be adjusted to yield better results as well as adjusting other aspects of the method, like a finer control on the flow rate of ACSF throughout the 21 hour incubation period through the use of pumps instead of gravity.

AIS length and number of the mild OGD slices didn't show any significant difference than control neither in pyramidal cells nor interneurons for CA1, CA3 and DG regions. A reason for this could be that four hour recovery is not enough for AIS lengths to change, and also it could be that interneurons suffered some injury from mild OGD that makes them unable to manifest AIS plasticity since they fire less even with increased excitation from pyramidal cells. Similarly, the AIS length or number of the CA1 pyramidal layer of the UVPS slices showed no difference than control slices, possibly due to cell death in both UVPS and control slices following 21-hour incubation. 20-minute OGD slices showed a significant decrease in AIS number compared to control slices which manifested itself after one hour of recovery and was significant after four hours of recovery. This could possibly be due to AIS proteolysis, which is known to be a sign of neuronal injury (Schafer et al., 2009) that manifests itself, although not immediately, following 20-minute OGD.

It is known that even after a mild period of cerebral ischemia (around 10 minutes), neuronal damage can occur which becomes evident after 3 days and the full extent of the damage of the ischemic insult takes a long recovery period to manifest itself (Mattson et al., 2000, Lai et al., 2014). Therefore, finding a method to extend the time that acute brain slices remain viable in a Haas-type interface chamber would be very beneficial to study the long-term effect of mild or strong OGD on the neuronal networks and single cell activity, as well as on cell death or on AIS morphology.

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