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Neurons control their output over long time scales to generate behavior. One well-studied mechanism thought to achieve network homeostasis, synaptic scaling, involves a uniform increase in excitatory synaptic strength to oppose reduced network activity. Although synaptic scaling is hypothesized to retain the normal balance of synaptic weights, quantitative scaling across synapses does not always occur, leaving to question the purpose of scaling on network function. We addressed this issue in the respiratory network of frogs, as synaptic compensation regulates respiratory motor function after inactivity associated with aquatic hibernation. Here we confirm that inactivity during hibernation elicits synaptic compensation in motoneurons, with mean increases comparable to shorter-term pharmacological inactivity. Despite similar amounts of compensation, pharmacological inactivity failed to scale synapses, but hibernators showed evidence of scaling that persisted even when motor activity has recovered. Blocking L-type Ca^{2+} channels after hibernation disrupted quantitative scaling of synaptic strength but did not impair compensation, uncoupling the scaling organization from compensation. Scaling *per se* played a vital role in regulating the network because respiratory motor outflow was weaker when neurons failed to scale upregulated synaptic weights. Thus, hibernation triggers synaptic scaling, and once activity restarts, an activity dependent Ca^{2+} signal maintains the scaling organization to regulate motor output. These results reveal that the organization of synaptic weights can play a distinct role from compensation in network homeostasis and show that compensation alone is not sufficient to prevent maladaptive network outputs following activity perturbations.

COMPENSATORY PLASTICITY VS SYNAPTIC SCALING; NOT ALWAYS ONE IN THE SAME

by

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APPROVAL PAGE

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CHAPTER I: INTRODUCTION

The brain is an essential organ of the body, it is responsible for maintaining activities such as sleeping, talking, walking, breathing, and much more. Neurons are the cells of the brain which are essential for maintaining these behaviors in the body. Neurons accomplish this goal by sending electrical signals along a chain of neurons until the signal reaches the target to elicit an action. In general, chemical signals are received to a neuron at the dendrites, the tree-like branches projections from the cell body. This signal then generates in the axon hillock or the initial segment and sends down the axon through an electrical signal called action potentials. Once the signal reaches the axon terminal, the electrical signal is sent out as a chemical signal in the form of neurotransmitters. The axon terminal ramifies against a postsynaptic neuron, and the signal is sent, and the process repeats in the next neurons (Figure 1).

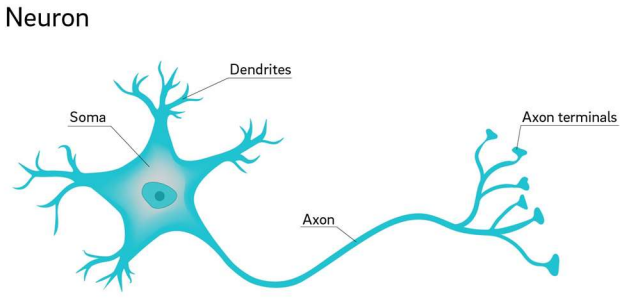

Figure 1: Cartoon Image of a Neuron, medicalxpress.com

This image depicts the basic structures of a neuron. Dendrite, where a chemical signal is received. Soma, the cell body. Axon, where the signal is sent along the neuron. Axon terminal, where the signal is released to be passed to an adjacent neuron, or postsynaptic neuron.

Depending on the type of neurons, different firing patterns can arise, which can lead to different behaviors. Some neurons fire fast, slow, rhythmically, or spontaneously in the absence of synaptic input. For example, neurons that cause automatic contraction of

the breathing muscles have quite different properties than sensory neurons that only fire input response to a specific stimulus, like a muscle contraction after touching a hot stove (Connors and Regehr 1996). However, the common properties among all the neuron types are the composition of synaptic receptors and ion channels that determine the electrical output of a neuron. The activity of synaptic receptors and ion channels must work synchronically to create appropriate patterns of electrical activity (Marder and Goaillard 2006). This is not a trivial problem for a neuron to handle. The receptors and channels are constantly turned over in the cell membrane, and changes in the environment can disrupt activity patterns, as well as trigger changes in channels and receptor expression (Marder and Prinz 2002). Thus, a fundamental question is, how do neurons maintain their activity patterns despite facing constantly changing conditions.

There are two general ways by which a neuron may maintain activity stability. First, the system may be “pre-programmed” to resist challenges to function outright. For example, one study in the crustaceans showed that even when exposed to a large range of temperatures, the pyloric network in the stomatogastric ganglion maintained its triphasic patterns of output. Second, neurons can actively regulate their function through compensatory mechanisms and feedback homeostasis. For example, one study in the *Drosophila* mushroom body showed that prolonged activation of the anterior paired lateral neuron leads to increased inhibition of neuronal activity to bring activity levels back to normal (Apostolopouloua and Lin 2020). It is vital that the brain maintains activity despite challenges. The inability to maintain functional activity levels through a disturbance can lead to the development of neurodegenerative, such as epilepsy and Alzheimer’s (Holmes and Ben-Ari 2001) (Tampellini 2005).

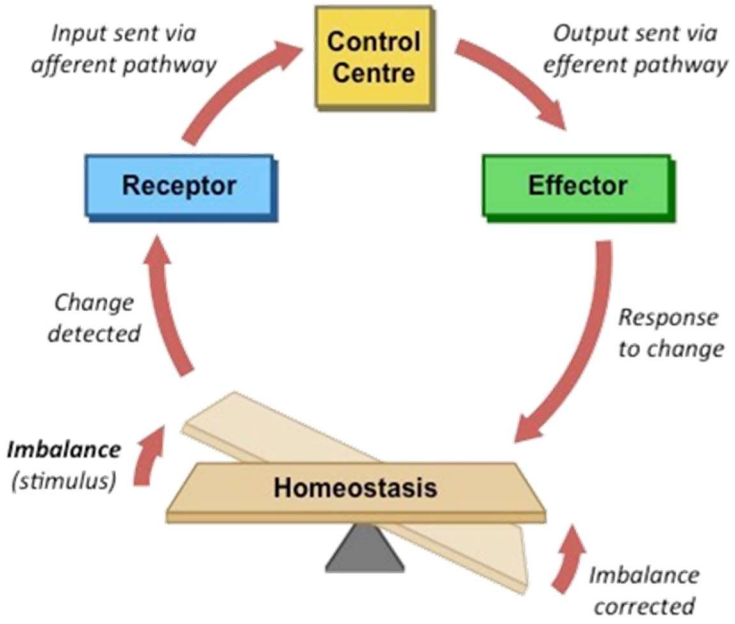

Figure 2: Homeostatic Plasticity Feedback Loop, Bioninja.com

This is a representation of the basic homeostatic feedback mechanism. When a biological variable is not stable, this multiple step mechanism brings the variable back into balance.

Compensation is the process by which neurons can oppose disturbances in activity stability, which follows a homeostatic feedback loop. The overall goal of this feedback loop is to maintain some feature of neural function (firing rate, network output, synaptic strength, etc.) within a defined range. The general view is that this feedback loop controls activity, by sensing variables associated to it, detecting when these variables deviate from a setpoint, and then triggering a compensatory response to bring the regulated variable back to baseline (Figure 2) (Turrigiano and Nelson 2004). This scheme is generally referred to as “homeostatic plasticity” because neurons modify their ion channels and receptors (hence plasticity) to regulate neuronal function (homeostatic). Therefore, homeostatic plasticity tends to oppose over-excitation or over-inhibition in neural circuits (Pozo and Goda 2010).

Within a neuron, there are many ions that are needed to maintain and sense activity, one being the cation calcium (Ca^{2+}). A major role of Ca^{2+} in a neuron is to act as a secondary messenger. Ca^{2+} is also thought to aid in signals for long term potentiation (LTP) and long-term depression (LTD). LTP is a preserved strengthening in synapses based on high amounts of activity between pre- and post-synaptic neuron, and LTD the opposite, a preserved weakening based on little activity (Brini, et. al 2014). In a computational study, the way Ca^{2+} signaling affect neuron activity was examined. A neuron will normalize its activity in response to available intracellular Ca^{2+}(O'Leary, et al., 2014) (Figure 3). It is thought that the Ca^{2+} signal senses a cell's activity levels, and its intracellular concentrations can be adjusted to alter excitability (O'Leary, et al., 2014).

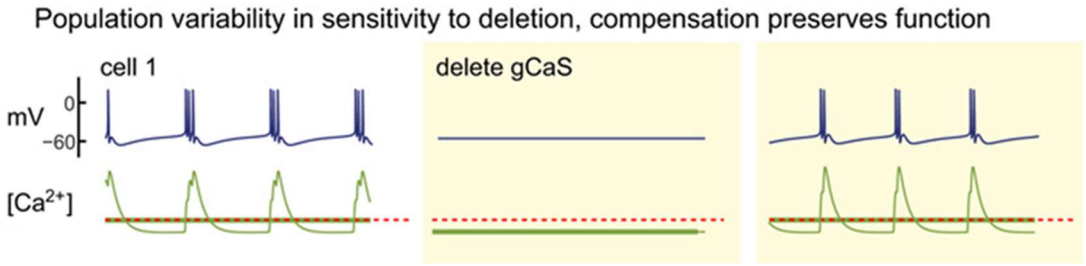

Figure 3: Ca^{2+} Signal and Neuronal Activity, from Computational Study, O'Leary, et al., 2014

Blue curve indicates cell activity by recording changed in mV. Green curve indicates average concentration of Ca^{2+}. Red dotted line represents target Ca^{2+} concentration. Left panel shows activity in untreated neurons when average Ca^{2+} matches target Ca^{2+}. Middle panel shows when average Ca^{2+} drops, so does activity. The right panel shows the compensation of activity following the acute depletion of Ca^{2+}.

Although several different mechanisms of homeostatic compensation exist in neurons, they can largely be broken down into two broad themes: synaptic and intrinsic. Synaptic compensatory plasticity involves adjusting the strength of synaptic drive within a circuit. For example, during chronic neuronal inhibition, neurons can increase their synaptic strength, and reduce synaptic strength during network hyperexcitation (Marder and Goaillard 2006). On the other hand, intrinsic plasticity involves adjusting the expression/function of ion channels along the neuron, making it more or less likely to fire action potentials.

Most of the work in this thesis focuses on postsynaptic homeostatic synaptic plasticity, through a mechanism referred to as “synaptic scaling.” During homeostatic plasticity, when synapses are strengthening, synaptic scaling is thought to occur. Synaptic scaling is the concept that during plasticity all synapses, across a neuron, increase at an equal amount (Turrigiano et al, 1998). The concept of synaptic scaling was first proposed in the late 1990’s, and in the last 20 years, studies have taken off in this area (Moulin et al 2020). Synaptic scaling is thought to follow the basic negative feedback loop (Figure 2) (Moulin et al, 2020).

In this study, mechanisms of compensatory responses in an *in vivo* model will be investigated. In the natural environment there are many factors that may impact the induction of compensatory mechanisms, such as activity drive in an organism, environmental temperature, the stage of development that the organism is in. It is also thought that compensatory mechanisms follow a rigid activity dependent negative feedback loop. The experiments described in this thesis involved combing and isolating different inducing factors of compensation to analyze the mechanism with the goal of identifying if compensation does in fact follow a rigid feedback loop.

The Model System:

Lithobates catesbeianus, the American Bullfrog, was used as the animal model. This animal model has a neuronal circuit that follows the homeostatic plasticity feedback loop, its breathing circuit. During most of its life, these amphibians depend on their lungs for gas exchange. The neuronal circuit that maintains respiratory activity is rhythmic. During the winter months, these animals will dive under water and maintain gas exchange only through their skin. This leads to a halt in activity within the motor neurons responsible for gas exchange controlled by lungs (Santin and Hartzler 2016). Electromyography recordings from bullfrogs’ respiratory muscles show in wintering conditions, the muscles of breathing are not in use, compared to warmer temperatures, when they are active (Santin and Hartzler 2017). In past studies, *in vivo* studies have shown that increases in synaptic strength are regulated through the homeostatic

mechanism, synaptic scaling, after three months of being in wintering conditions to maintain appropriate motor outflow from the network (Santin, et. al. 2017).

This model has two major advantages. First, this wintering condition induces a naturally occurring homeostatic response following activity inhibition. Second, since the changes due to the homeostatic response appear to regulate behaviorally relevant circuit homeostatic plasticity in a naturalistic model can be studied. Most studies of homeostatic plasticity and compensation are in a model that does not occur naturally, for example, knockout mouse models (Wenner and Bülow, 2019). With the bullfrog, the connection between a natural challenge in activity and behavior changes can be studied in a more natural way.

CHAPTER II: AIMS

Aim one: Determine a time course of synaptic plasticity in wintering frogs

In this aim, I tested the hypothesis that synaptic compensation increases gradually vs. saturating during winter. As most work in synaptic compensation has been performed in cell culture models and/or with activity perturbations that are not a true reflection of the animal's life history; thus, a major gap in the field has been in understanding how compensatory regulation is controlled across time in the intact brain with a physiological perturbation for the animal.

By looking at multiple stages of a disturbance *in vivo*, we can observe how neurons in living animals respond, and better understand how the brain tracks its activity pattern. Spontaneous excitatory post-synaptic activity was analyzed to determine the synaptic strength and cell excitability. An increase in synaptic strength and cell excitability implies that the neurons compensate for the lack of activity following the winter simulation. This follows the homeostatic plasticity loop because neurons can sense and compensate for the change following a disturbance of activity.

As most work in synaptic compensation has been performed in cell culture models and/or with activity perturbations that are not typical of the animal's life history, a major gap in the field has been in understanding how compensatory regulation is controlled across time in the intact brain with a physiological perturbation for the animal.

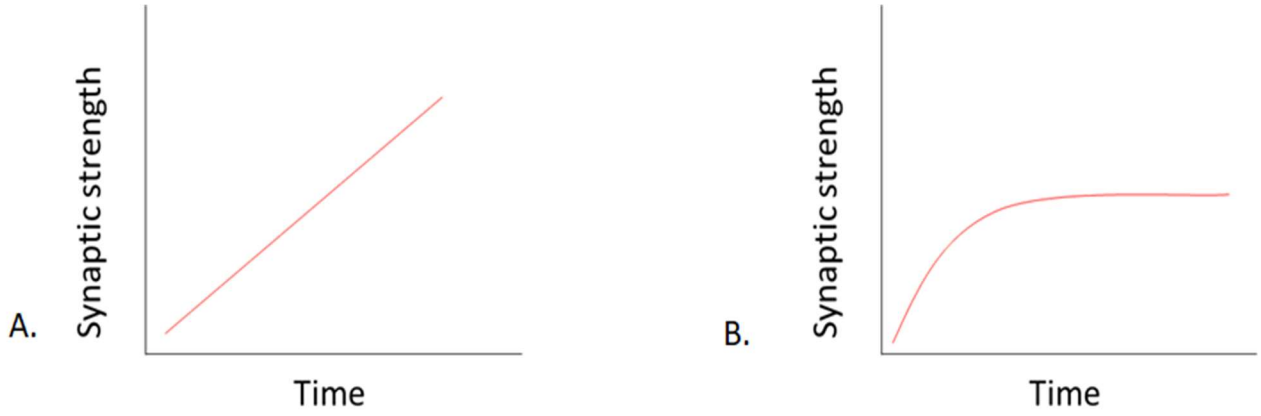

Figure 4: Possible Outcomes for Synaptic Plasticity in Time Course Experiment

(A) Possible outcome for aim one, a linear relationship between time spent in in activity and increases in synaptic strength. (B) Another outcome of aim one, after a certain amount of time of inactivity, there will be a saturation of synaptic strength.

Synaptic strength will be compared in three different wintering groups, control (warm acclimated), two weeks, and one month in winter simulation. This experiment will determine the length of time that frogs need to remain in wintering conditions before compensation occurs, which was previously unknown. By identifying a time course of plasticity, insight to the regulation scheme can be made. It will show if there is a quick onset of the homeostatic mechanisms to “rebalance” the system following the disturbance. On the other hand, it could show if there is a continuous induction of homeostatic mechanisms over a long period of time (Figure 4).

Aim two: Identify whether inactivity induces synaptic compensation and scaling in response to overwintering

A known voltage-gated sodium channel blocker, tetrodotoxin (TTX), silences neurons, and will therefore be used to induce inactivity pharmacologically. And another blocker, DNQX, inhibits AMPA-glutamate receptors, and therefore, reduces activity in neuronal systems. Our goal here is to compare the synaptic response elicited by altering activity pharmacologically to that induced by the inactivity experienced by frogs in the winter. For my thesis project I determined whether circuits from overwintered animals with and

without TTX-induced silencing elicited elicit further compensation or saturation. This experimental design allowed us to determine whether stimuli that induce responses that mimic the natural response to winter inactivity and then whether winter response occludes the response to further inactivity induced by TTX. In addition, we can determine whether each perturbation results in scaling of the compensatory increase in synaptic strength. The third component to be investigated will be blocking the L-type calcium channel into the neuron with Nimodipine (NIMO) in winter animals. The experimental approaches will be described in detail in the results section. Miniature excitatory postsynaptic activity and firing properties will be analyzed to compare synaptic strength.

CHAPTER III: METHODS

Animal Model

Bullfrogs were purchased from the Rana Ranch (Twin Falls, ID, USA) and were maintained in animal housing until experiments as described by Santin, et. al. 2017 elife. Control frogs were maintained at room temperature, about 20 ºC, and provided aerated water, with wet and dry areas within the take. For the winter frogs, they were also provided aerated water with the same chemical compositions. The temperature of water in wintering frogs was lowered from 20ºC to 2ºC over the course of a week, dropping about 2-3 degrees per day. During the experiment, all frogs were kept on a 12 hr:12 hr light: dark cycle. Frog care and removal occurred during light hours. These experiments have IACUC approval. For aim one, wintering frogs will be used at different time points of cold conditions; one week, two weeks, and one month. For aim two, wintering frogs will be used at one month.

Dissection

To obtain the brainstem from the bullfrogs, the frogs were removed from the cage, sedated by inhalation of isoflurane (approximately 1 ml per liter), and rapidly decapitated. The dissection was performed using chilled bullfrog artificial cerebrospinal fluid (aCSF; concentrations in [mM]: 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 7.5 glucose, 40$NaHCO_3$, 2.5 $CaCl_2$ and 1 NaH_2PO_4, and gassed with 90% O_2, 1.3%CO_2, balance N_2; pH = 7.8;CO_2/pH values reflect normal for bullfrogs), constantly bubbled with 98.5%/1.5% CO_2. The midbrain and rostral spinal cord were removed, while ensuring the cranial and spinal nerves were exposed (Figure 5) (Santin, et. al. 2017).

Figure 5: Image of Dissected Frog Brain, with Vagus Nerve Circled

In a healthy dissected frog brain, cranial and spinal nerves are clearly visible, making it easy to identify the 4th root of the vagus nerve, circled.

For brains used in aim one, immediately following the dissection, brain tissue was prepared for single cell electrical recordings. For brains used in aim two, 16-hour extracellular recordings were made to track activity following addition of different pharmacological drugs to the network prior to tissue preparation for recordings. A glass electrode recoded activity from the exposed vague nerve on LabChart 8 (ADinstruments, Sydney, Australia). Burst frequency, rise time, burst with, and decay time were analyzed on LabChart 8.

Tissue Preparation

Following the dissection, the brain was super-fused in oxygenated aCSF with a flow rate of ~5-7 mL per minute. The laryngeal branch of the vagus nerve was isolated and then backfilled with a TRITC dextran 3000 MW, dye (Life Technologies Corporation, Eugene, OR USA). To visualize motor neuron cell bodies, about 1 uL of 10% dye was loaded into the tip of a glass pipette that fit snuggly around the cut 4th root of the vagus, as this nerve contains axons that most innervate the glottal dilator, a respiratory muscle in

anuran amphibians (Gans et al., 1969). Backfills lasted two hours and enabled robust identification of labeled neurons for electrophysiological recording.

Following the dye loading process, tissue was sliced with a vibratome at a 300-uM thickness, approximately 300-900uM rostral of the hypoglossal nerve. Tissue slices were left to recover for an hour before being transferred to the electrophysiology rig for electrophysiology recordings (Figure 6) (Santin, et. al. 2017).

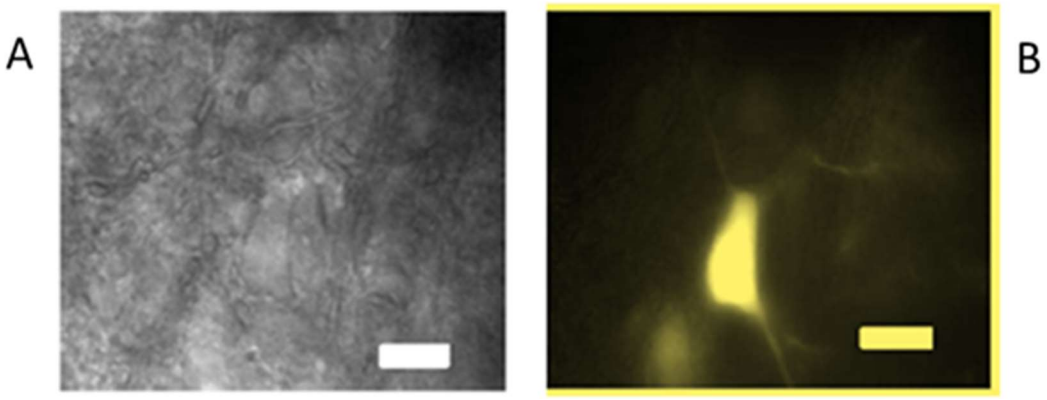

Figure 6: Images of Motor Neurons, Santin, et. al. 2017

Microscopic images following the fluorescent dye loading into the fourth root of the vagus nerve. (A) Image of tissue in the bright field at 60x magnification. (B) Image of tissue in the florescent field at 60x magnification. Scale bar measures 20 μm.

Whole-cell patch clamp electrophysiology recordings

Synaptic activity was assessed using whole-cell patch clamp on Clampex 10 (Molecular Devices, LLC, San Jose, CA) on motor neurons in brain slices. To identify the time course of plasticity spontaneous excitatory postsynaptic currents (sEPSC) was recorded at two-time groups two weeks and one month in wintering conditions. sEPCS are voltage clamp recordings to measure the strength of a synapse by recording the current received by a post-synaptic neuron. Voltage is held at -80mV, and a 10 mV step up is taken to record input resistance and access resistance. The recorded current is a result of spontaneous vesicle release, with neurotransmitter, and vesicle release resulting from action potentials.

For the mechanism experiments miniature excitatory postsynaptic currents (mEPSC) are recorded. The mEPSCs are similar recordings to sEPSCs except mEPSC recordings are only from spontaneous vesicle release; this is done by blocking voltage gated sodium channels with tetrodotoxin, a pharmacological drug. Sodium channels are necessary for action potentials to fire, by elimination of action potential, it removes signals due to action potential from the recording.

In the sEPSCs and mEPSCs the amplitude of each event, area under the curve of each event, and frequency of synaptic events were analyzed. A higher frequency suggests a greater number of vesicle release across a synapse, filled with neurotransmitters, from the presynaptic neuron to the post-synaptic neuron. An increased amplitude indicates a higher sensitivity of the post-synaptic neuron (Han and Stevens 2009).

To assess the cell's health, current clamp recordings were made to measure the resting membrane potential (RMP) of the cell. Cells with very positive RMPs will be considered unhealthy and not included in the data set.

Drugs

Tetrodotoxin (TTX) is a drug that binds and blocks voltage-gated sodium channels. During an action potential, these channels open during the depolarization stage, if inhibited action potentials stop (Lago, et. al. 2015). TTX will be used in two different ways, to silence activity and induce compensation, and to record mEPSCs in all experimental groups. To silence activity 10 nM TTX will be used for 16 hours to induce compensation. To record mEPSCs 500 uM TTX will be used for approximately 3 minutes during whole cell recordings (Santin, et. al. 2017).

6,7-dinitroquinoxaline-2,3-dione (DNQX) is a glutamate receptor antagonist. Glutamate receptors are located on the post-synaptic neuron and bind neurotransmitters and help regulate excitatory synaptic transmission. As an antagonist, DNQX will lead to a halt in activity (Traynelis, et al. 2010). This activity silence will test synaptic plasticity through receptor blocking and will be done by superfusing the brain in 10 mM for 16 hours.

Nimodipine is a L-type calcium channel antagonist. These L-type calcium channels are voltage gated channels that are expressed throughout many cell types in the body. In neurons, these channels allow the influx of Ca2+ during an action potential. Working as an allosteric inhibitor, Nimodipine, can halt the influx of Ca2+ into the neurons, blocking activity (Carlson, et al. 2020). This activity silence will test synaptic plasticity through calcium sensing and will be done by superfusing the brain in 10uM for 16 hours.

Data Analysis

For sEPSCs and mEPSCs amplitude measurements, access resistance, and input resistance will be analyzed in voltage clamp on LabChart 8. Amplitude is measured from the distance between the baseline of the recording to the peak of an event. To find the amplitude of a cell, events were recorded for approximately 30 second, and an average amplitude was calculated (Figure 7). Electrical noise was excluded from the events by implementing these restraints; minimum height 7.5 pA, maximum rise time 10 ms, and minimum tau 5 ms.

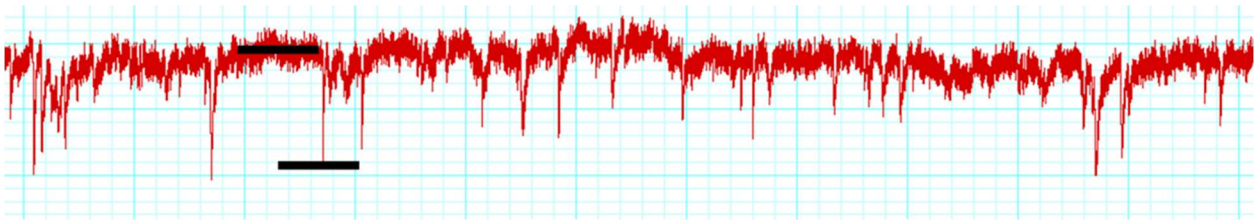

Figure 7: Raw Recording of Voltage Clamp

A raw recording of voltage clamp amplitude is recorded by measuring the difference between baseline (top black line) and peak of event (bottom black line).

Input and series resistances were calculated by making a 10-mV step, from -80 mV to -70 mV. Input resistance (Ri) tells us the resistance of the circuit being recorded. In these experiments the circuit is the cell, and Ri is calculated by, Ri= (Baseline-instantaneous stead state) *1000. Series resistance (Rs) tells us how strong of a connection our recording petite has to a cell and is calculated by, Rs= (baseline-step peak) *1000. Any cell recording with a greater than 25MΩ series resistance was not included in the data set.

Resting membrane potential will be analyzed in current clamp on Clampfit 10.7 for sEPSCs and mEPSCs. To get these recordings, no current is injected into the cell (Figure 8). Cell's membrane potential generally reneged between -45mV and -6mV, cells that were visibly unhealthy were above -45 mV and were removed from the data set.

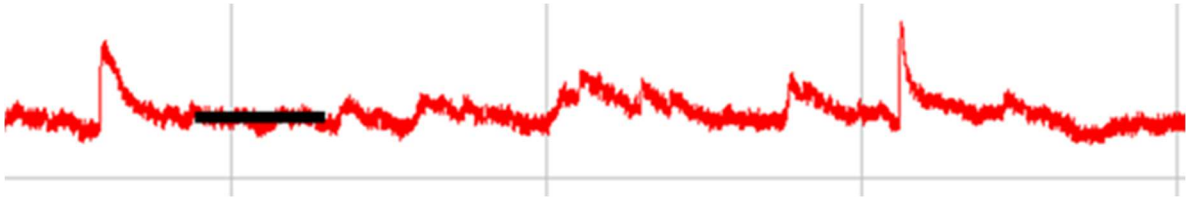

Figure 8: Raw Recording of Current Clamp

Raw current clamp recording can measure membrane potential in mV (black line).

A raw recording on membrane potential in current clamp, shows where membrane potential is calculated (black line).

Statistics

Data were analyzed and plotted using GraphPad Prism (GraphPad, Software, San Diego, CA). All data were cleaned for outliers prior to analysis using the Grubb's test, allowing one outlier to be identified per group. Analyses between two groups were performed using a two-tailed unpaired t test. When standard deviations were different between groups, a Welch's test was used. Analyses involving three or more groups were performed using a one-way ANOVA and Holm-Sidak's multiple comparisons test. To infer synaptic scaling, a series of 50 mEPSCs were chosen per cell and the entire distribution was ranked from lowest to highest for control and treatment conditions. Ranking the distribution of mEPSCs requires the same number of cells per condition, or there would be a different number of mEPSCs per group which would skew the ranking. When ranking the data where control and treatment conditions had different numbers of cells (e.g., 16 in the control vs. 17 in TTX), one cell was randomly removed for the scaling analysis. We did this by assigning a number to each data point (e.g., 1-17), and then used a random number generator to choose which cell to remove the cell for scaling analysis. This allowed an equal number of mESPCs to be ranked and scaled in

each group and removed potential investigator bias associated with removing the cell. Ranked data were plotted on an x-y graph and then fit by a linear regression to obtain the slope of the line. Each point in the treatment distribution was then mathematically downscaled by the slope of the line from the linear regression produced by the rank ordering of the two distributions and plotted as cumulative distributions. When comparing two cumulative distributions, the Kolmogorov Smirnov test was run. Significance was accepted at $p<0.05$.

CHAPTER IV: RESULTS

Cells used for this study were healthy, with basic physiological/technical properties showing no differences across groups.

To ensure physiological recordings were only made on healthy cells, as well as cells that were indeed respiratory neurons, many precautions were taken. First, cells were measured in length under the bright field, cells smaller than 25-um were not recorded from. Next, in voltage clamp, input and series resistances were calculated, description in methods section.

In the current clamp recordings, there was no significant difference in membrane potential values (in mV) between all groups (Figure 9 A). In the voltage clamp recordings, there was no significant difference in input resistance or series resistance (Figure 9 B) (Figure 9 C). These recordings show that cells had a healthy resting membrane potential for proper activity, had a high enough resistance to be selectively permeable to ions crossing the membrane, and that was a strong connection between pipette and cell for good electrical recordings.

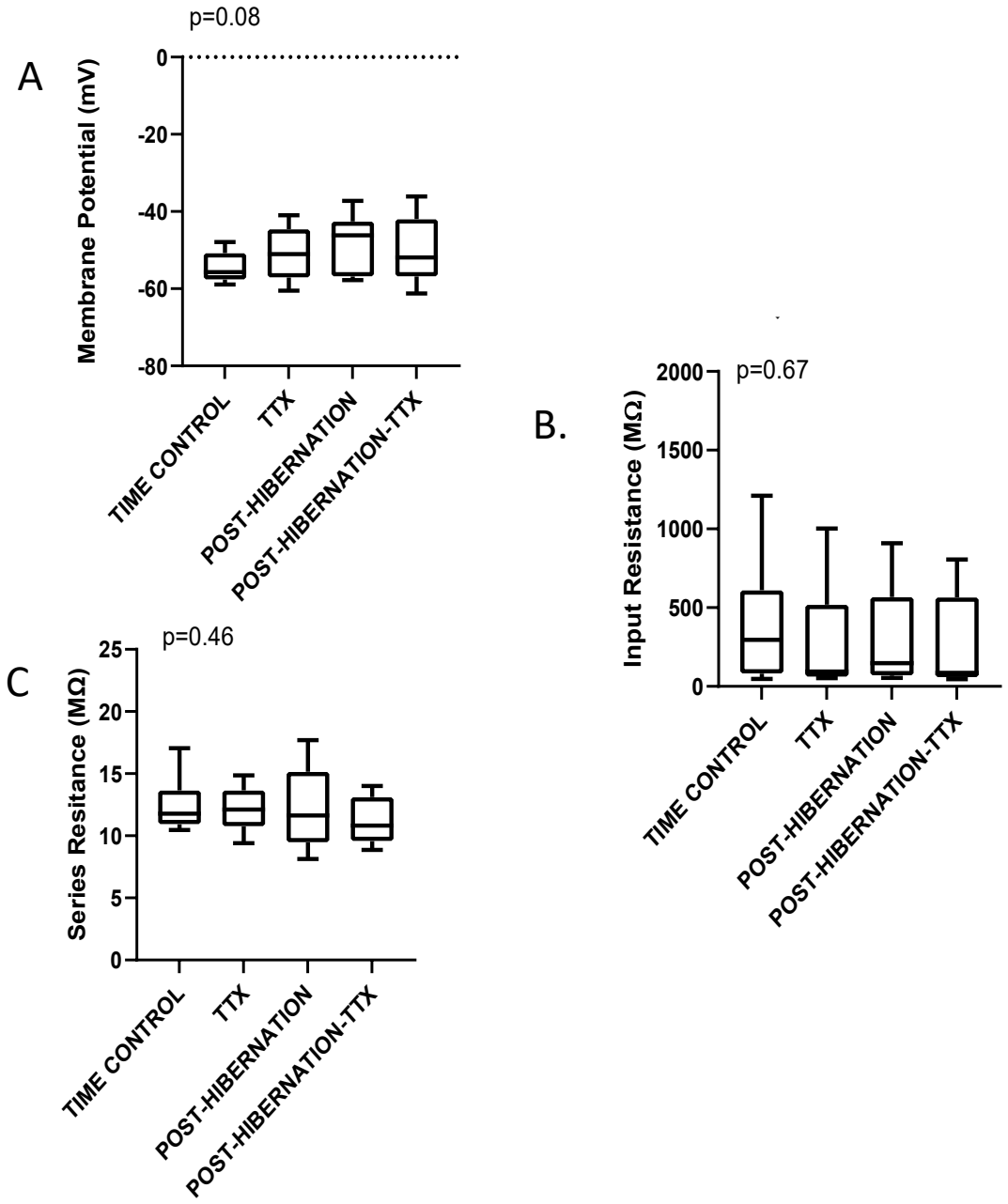

Figure 9: Membrane Potential, Input Resistance, and Series Resistance for each Experimental Group

(A) Box and whiskers plot of membrane potential in all experimental groups, time control (warm acclimated) n=18, TTX (warm acclimated with 16-hour TTX

exposure) n=19, post-hibernation (1 month in cold acclimation) n=17, post-hibernation-TTX (1 month cold with 16-hour ttx exposure) n=16. Shows no significance in any group. (B) Box and whiskers plot of input resistance in all experimental groups. (C) Box and whiskers plot of series resistance in all experimental groups. Shows no significant difference.

Synaptic strength saturates after wo weeks of inactivity

To identify the amount of time needed for inactivity in motor neurons to induce a compensatory response sEPSC are recorded at three different temperature groups: in control cells, two weeks cold acclimated, and four weeks cold acclimated (Figure 10). The significant difference between control cells and two weeks cold acclimated, and the lack of significant difference between two and four weeks cold acclimated show the synaptic strength compensation saturates following about two weeks of inactivity. This experiment set up the rational for the following experimental designs, frogs were left in cold acclimation for four weeks in continuing experiments. This is because by four weeks it appears that compensation has occurred (Figure 10).

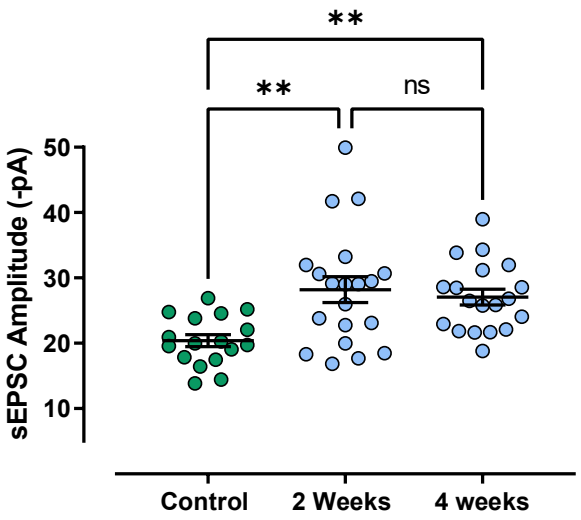

Figure 10: Saturation of Synaptic Strength Following 4 Weeks of Winter.

Showing individual data points along with average sEPSC amplitude (-pA) for each experimental group; Control (warm acclimated) n=17, 2 weeks (2 weeks cold acclimated) n=20, and 4 weeks (4 weeks cold acclimated) n=19.

Inactivity due to wintering conditions leads to synaptic compensation.

To investigate compensation through different induction mechanisms. Time control mEPSC were compared to two other groups; one month in cold acclimation and warm acclimated with treated with sodium blocker TTX for 16 hours, (Figure 11 A and B). Another comparison was made between one month cold acclimated with one month cold acclimated with 16 hours of TTX treatment (Figure 11 C). Data is shown in cumulative distribution plots, ranking all synaptic events (left), as well as a graph showing each cell's average mEPSC amplitude (right).

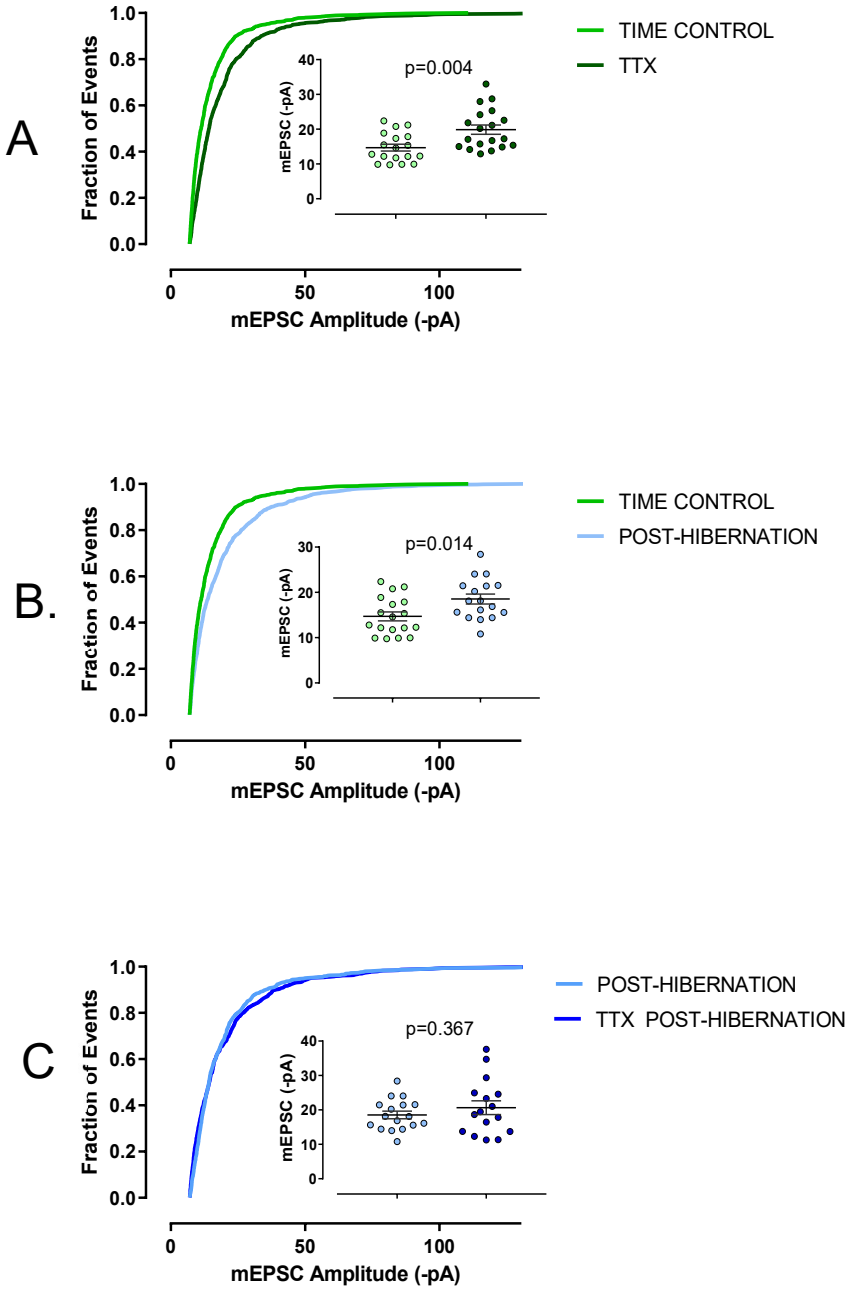

Figure 11: Ranking of Synaptic Events in Wintering and Activity blocked Neurons.

Fraction of events was ranked by amplitude comparing (A) time control (warm acclimated) n=18 vs TTX (warm acclimated with TTX treatment) n=19, (B) time control vs post-hibernation (one month cold acclimated) n=17, and (C) post Hibernation vs TTX post hibernation (one month cold acclimated with TTX treatment) n=16.

To identify if synaptic scaling occurs within any experimental group, a scaled plot needs to be calculated, as stated in the methods. Three scaled plots are created, time control compared to three groups: warm acclimated treated with sodium blocker TTX for 16 hours, one month in cold acclimation, and one month cold acclimated with 16 hours of TTX treatment (Figure 12).

When ranking the events into scaling, time control was compared to three groups; warm acclimated with treated with sodium blocker TTX for 16 hours, one month in cold acclimation, and one month cold acclimated with 16-hour of TTX treatment (Figure 12).

The only group comparison that showed no synaptic scaling was time control vs TTX (Figure 12).

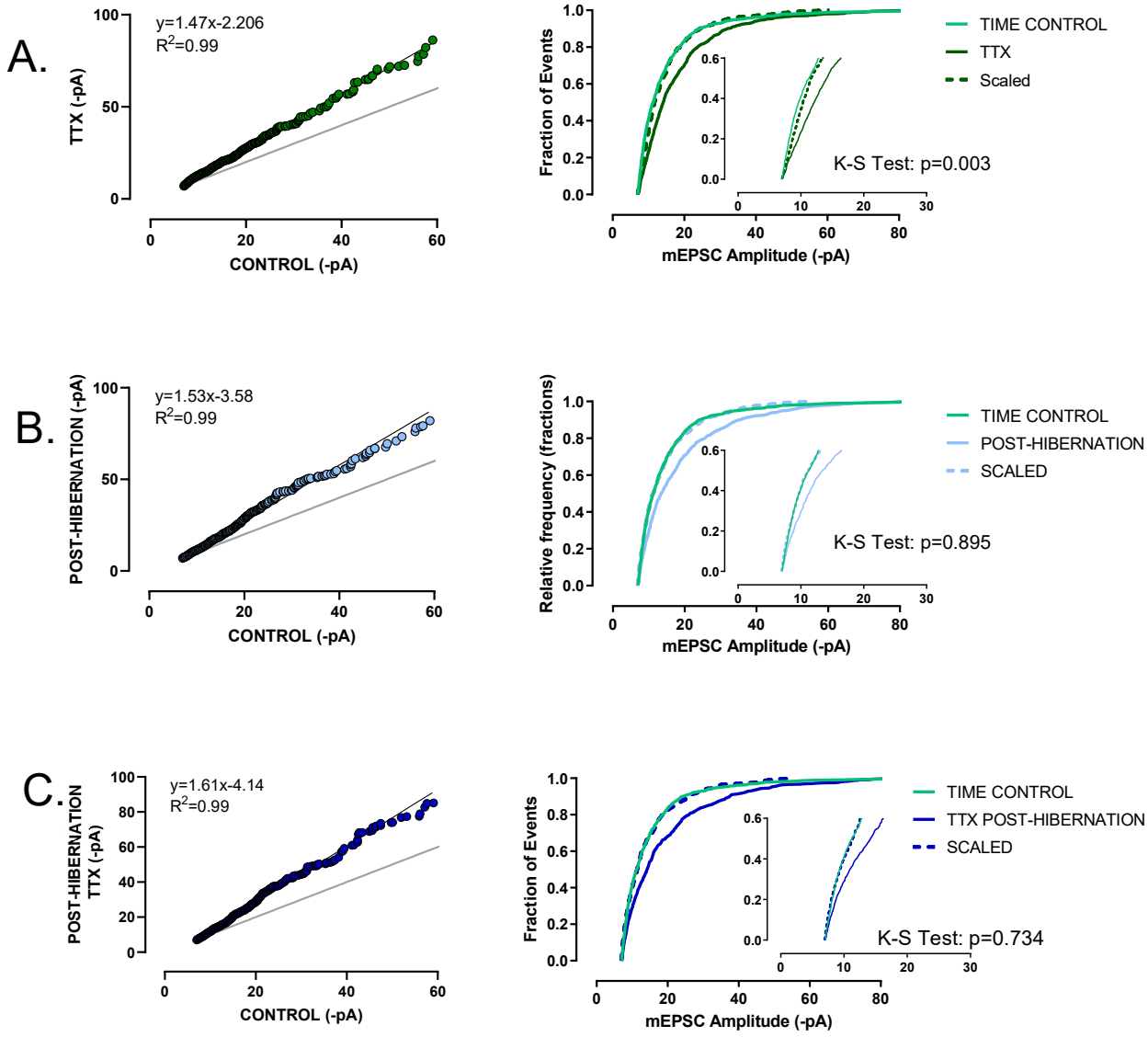

Figure 12: Determination of Scaling in Wintering and Activity Blocked Neurons

Determination of scaling between: (A) time control (warm acclimated with 16 hours of no drug exposure) n=18 vs TTX (warm acclimated with 16 hours of TTX exposure) n=19, (B) time control and post-hibernation (one month cold acclimated with 16 hours of no drug exposure) n=17, and (C) time control and TTX post-hibernation (one month cold acclimated with 16 hours of TTX exposure) n=16. Scaling was calculated by the ranking

of amplitude between (A) Time control vs TTX (B) Time control vs post-hibernation and (C) Time control vs TTX post hibernation. K-S number compares Time control plot with scaled plot.

Activity needs to be silenced to induce synaptic plasticity

Animal exposure to hibernation conditions, as well as tissue exposure to TTX, leads to a halt in circuit activity. An activity reducer, DNQX**,** an AMPA receptor blocker was used to test if activity reduction was enough to induce compensatory responses. Over 16 hours DNQX reduced extracellular circuit activity by an average of 88% (n=6); activity in 2 reduced by 100%, and the rest 80%, 95%, 85%, and 71%. This reduction in activity, in activity shows no effect on synaptic strength (Figure 13). This supports the idea that for compensation to occur through activity blockers, activity needs to be completely blocked.

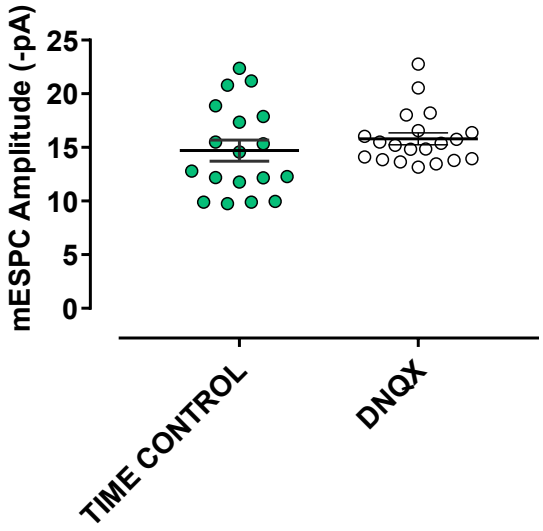

Figure 13: mEPSC Amplitude Control Compared to TTX Treated Neurons

The mEPSC amplitude is compared in time control (warm acclimated) cells n=18 and DNQX (warm acclimated with DNQX treatment) cells n=20. Showed no significant difference between two groups.

Block of calcium signal unscales synaptic compensation

Ca^{2+} is an important signaling molecule in neuronal biochemical pathways and plays a key role in coordinating channel and receptor organization in neurons (Santin & Schulz, 2019). Thus, we wanted to access whether synaptic compensation and scaling are maintained after overwintering through calcium-sensitive feedback, and then to do determine the impacts on network function. Here, cold acclimated frogs were also treated with NIMO, showing a significant difference in synaptic strength (Figure 14 A). However, this compensation does not follow the mechanism of synaptic scaling (Figure 14 B & C)

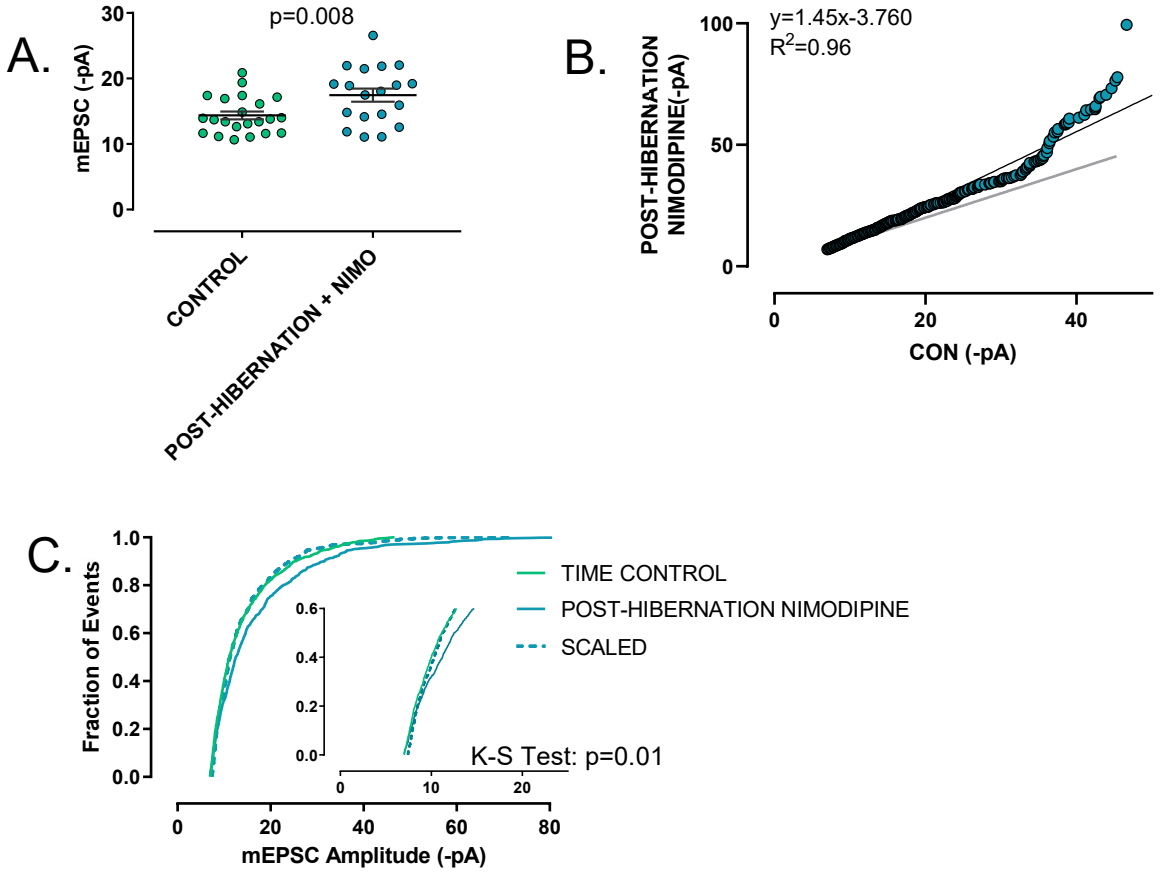

Figure 14: mEPSC Amplitude Control Compared to Post hibernation Nimodipine Treated Neurons, With Scaling Calculations

(A) The mEPSC of control (warm acclimated with 16 hours of no drug exposure) n=21 and post hibernation +NIMO (one month cold with 16 hours of nimodipine exposure) n=19 significant difference in group. (B) Scaling curve calculated between Control and post hibernation +NIMO. (C) Fraction of event plot scaling plot with control, post hibernation +NIMO, and scaled plot.

Calcium signal blocking induces characteristic changes in extracellular circuit recordings

Since compensation was induced following wintering conditions and 16-hour NIMO treatment, but it was not scaling, we wanted to assess the impact of this on the function of those cells in their intact network. Therefore, we recorded the activities of these motor neurons in response to physiological inputs and compared them to output properties among time control, post-hibernation (synaptic compensation + scaling), and post-hibernation+ nimodipine (synaptic compensation + no scaling): burst frequency, rise time, bust width, and decay time. Burst frequency and rise time showed no difference (Figure 15) (Figure 16 A & B). However, there was a significant difference in burst width and decay time (Figure 15) (Figure 16 C & D).

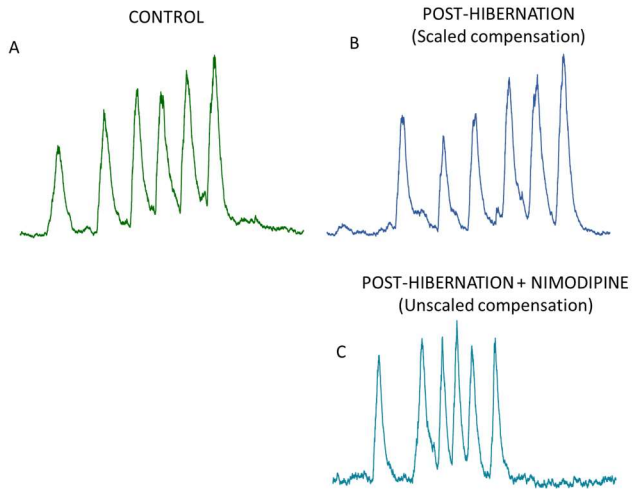

Figure 15: Raw Traces of Extracellular Activity

Raw traces from extracellular activity from (A) time control, (B) post hibernation, and (C) post hibernation with nimodipine exposure

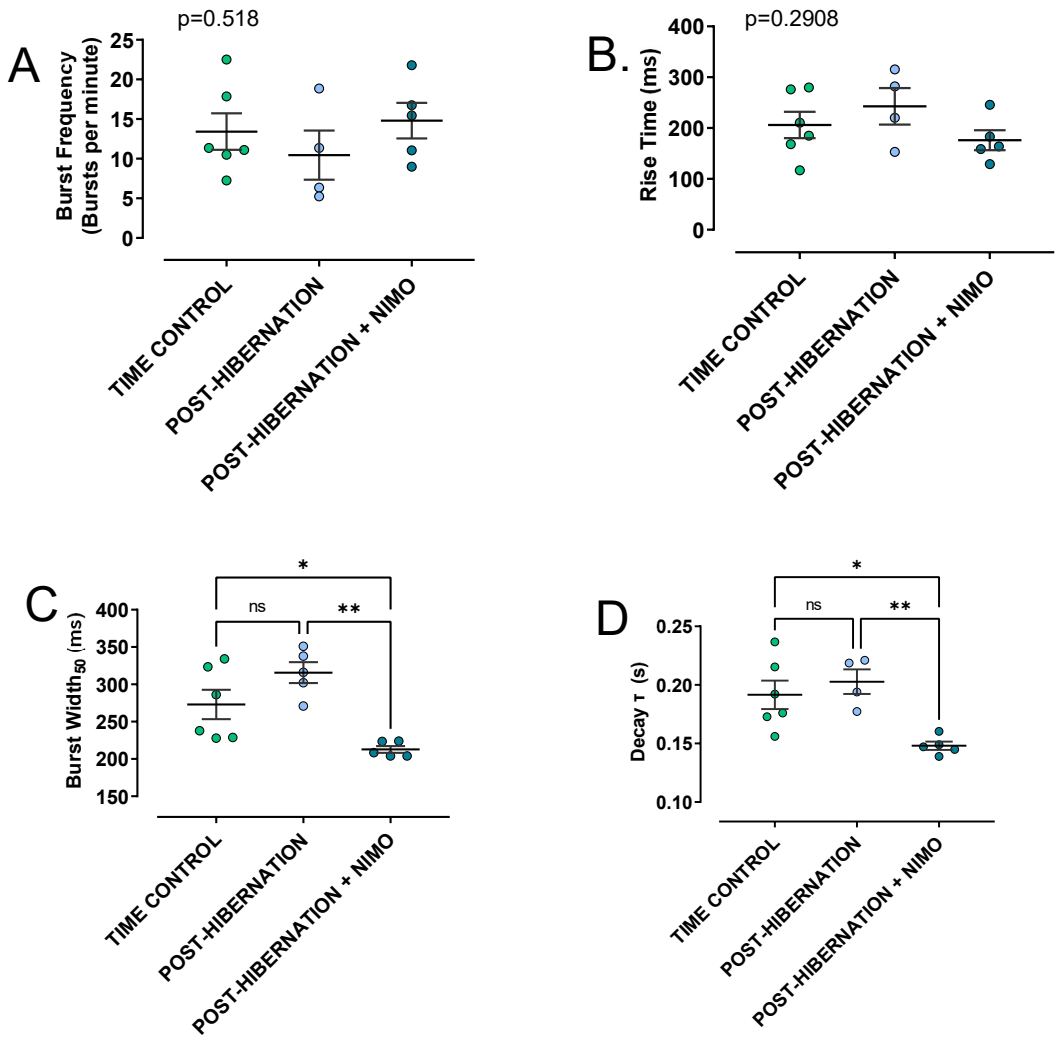

Figure 16: Changes in Characteristics of Electrical Circuit Events Following Calcium Signal Blocker

(A) The burst frequency of experimental groups; time control (warm acclimated with 16 hours of no drug exposure) n=6, post hibernation (1 month cold acclimated with no drug treatment) n=4, and post hibernation + NIMO (1 month cold with 16 hours of nimodipine exposure) n=5. (B) The rise time of experimental groups (C) Burst width of experimental groups. (D) The decay time of experimental groups.

These results suggest that synaptic scaling *per se* regulates motor output properties independent of total synaptic strength. However, it is possible that reduced duration and

decay time are a result of the direct block calcium channels by nimodipine and not the effects of nimodipine on “descaling” synaptic weights. To assess this, we looked at acute vs. chronic effects of nimodipine on burst width and decay time. Time control and post-hibernation preparations maintain stable burst properties for the entire protocol. For post-hibernation tissue with 16 hours of nimodipine, acute exposure to nimodipine does not affect burst properties; however, there is a drop in burst width and decay time with chronic exposure. These data imply that burst properties are not affected by acute block of calcium channels and argue for an effect of calcium block on disrupting scaling organization (Figure 17 A) (Figure 17 B).

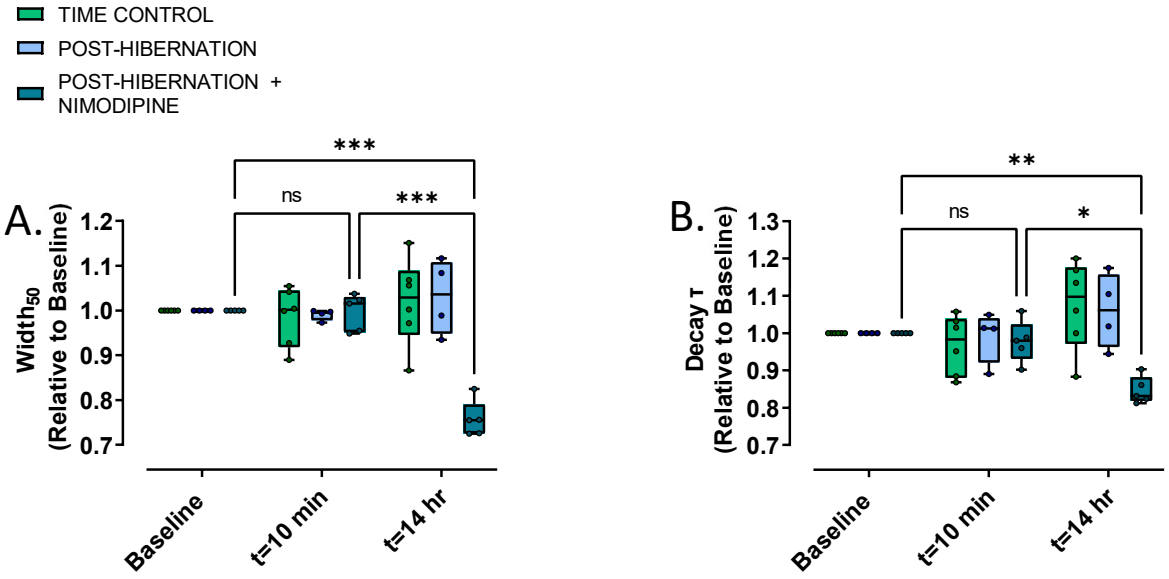

Figure 17: Changes in Characteristics of Single Extracellular events Following Calcium Signal Blocker

(A) The single extracellular event width following 10 minutes of activity and 14 hours of activity, change is compared to baseline activity, in experimental groups; Time control (Warm acclimated with 16 hours of no drug exposure) n=6, post-Hibernation (Cold acclimated with 16 hours of no drug exposure) n=4, and post hibernation + nimodipine (1 month cold with 16 hours of nimodipine exposure) n=5. (B) The single extracellular event decay time following 10 minutes of activity and 14 hours of activity, change is compared to baseline activity, in experimental groups.

Nimodipine does not change any other characteristics of activity

It is also possible that blocking L-type calcium channels has effects on other neurophysiological properties that could affect burst properties. To identify if the changes in burst properties are likely to occur by disrupting synaptic scaling were due to the NIMO treatment and or other changing factors, the membrane potential, input resistance, firing rate (at 3X recruitment threshold), mEPSC frequency were analyzed (Figure 18). No analyzed characteristics were significantly different on any groups.

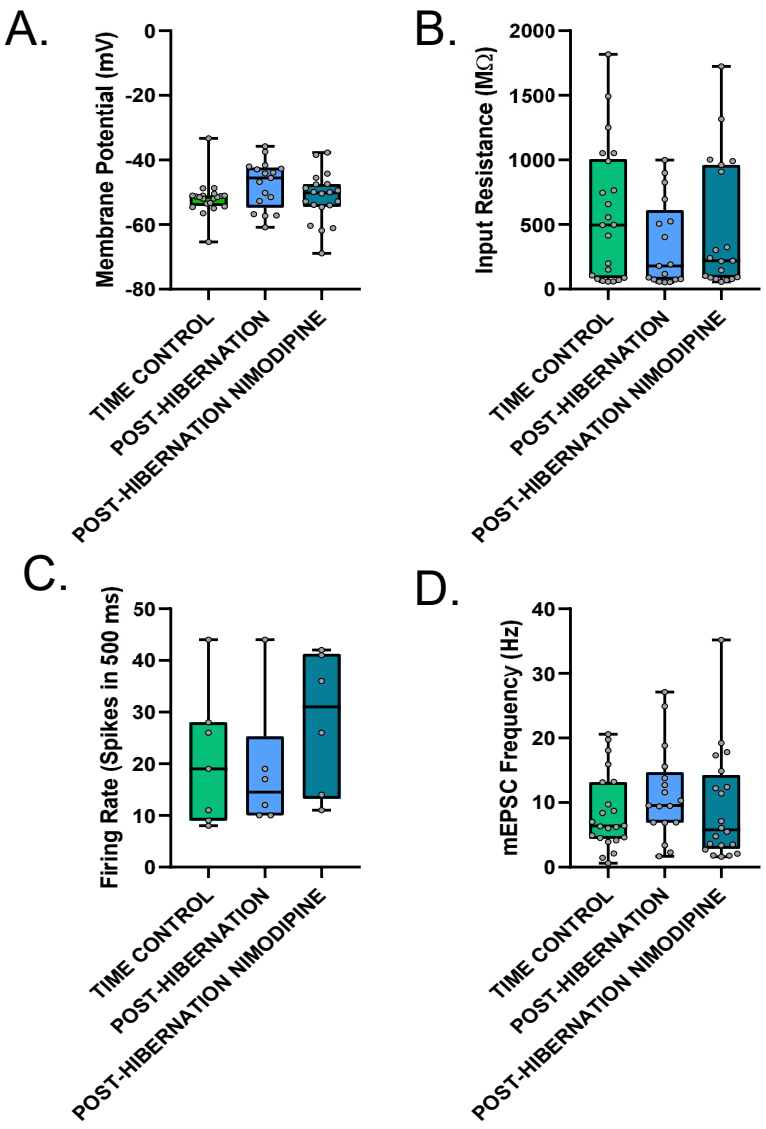

Figure 18: Characteristics of Neurons in Data set

(A) The membrane potential in experimental groups; time control (Warm acclimated with 16 hours of no drug exposure) n=6, Post hibernation (1 month cold acclimated with 16 hours of no drug exposure) n=4, and post hibernation nimodipine (1 month cold acclimated with 16 hours of nimodipine exposer) n=5. (B) The input resistance in experimental groups (C) The firing rate of experimental groups. (D) The mEPSC of experimental groups.

CHAPTER V: DISCUSSION

Neuron function is important for maintaining many process and activities on the body. Like almost any biological process, there can be disturbances in the activity of a neuron. These disturbances can result from an array of situations; a change in physical environment (temperature), changes in chemical environment (e.g., pH or neuromodulators), or changing activity levels (learning and development). Neurons can sense changes in activity, alter synaptic and intrinsic properties, and alter the output of activity back to “baseline” activity though homeostatic plasticity. This plasticity follows a general homeostatic feedback loop (Figure 2).

A widely studied mechanism of homeostatic plasticity is synaptic scaling. Synaptic scaling is the concept that each dendritic synapse within a neuron, will increase or decrease at the same amount to stabilize activity rates in a neuron (Turrigiano 2008). Synaptic scaling is often studied in neuronal cell lines with pharmacological activity inhibition, predominantly TTX. There are also studies done in mouse models where the visual cortex is inhibited by damage to the brain or light deprivation. Many of these synaptic scaling models do not include synaptic scaling in a setting that occurs naturally.

For my thesis research, I used the same animal model as in a 2017 study by Santin, et al., to study synaptic scaling. However, there were differences between my studies and those described it the Santin. et al 2017 paper with respect to acclimation to the cold time and time spent in hibernation conditions. The 2017 study lowered water temperatures from room temperature to 2°C over the course of six weeks, and for my thesis research temperatures were brought to 2°C over the course of one week (Santin, et al 2017). Regarding time spent in hibernating conditions, before this thesis research was done these was no previous work on the amount of time in inactivity would induce compensatory mechanisms in this animal model. This project suggests only 4 weeks in hibernation conditions is needed, compared to three months (Santin, et al 2017) (Figure

10). In these experiments, it is shown that pharmacological blockade of activity, through TTX exposure is not enough to induce synaptic scaling. There is an unknown synaptic scaling trigger that is only occurring during the cold simulation and not in philological activity block. This is surprising because TTX is one of the most common pharmacological drugs used to study synaptic scaling.

While this study investigated the triggers to synaptic scaling with the exposure of post hibernation neurons to nimodipine, it also shows the uncoupling of synaptic scaling and another form of compensation (Figure 14). There are two major pathways of plasticity that are studied in this field: synaptic scaling and Hebbian plasticity. Hebbian plasticity involves alteration in individual dendrites receiving input, while synaptic scaling involves alterations in all dendrites across the neuron (Pozo and Goda 2010). Major pathways of Hebbian plasticity include LTP and LTD, which Ca^{2+} plays a key role (Brini, et al 2014). This Hebbian plasticity may be induced with the block of Ca^{2+} signal, but more studies on this need to be done.

CHAPTER VI: CONCLUSION

The first major findings of these experiments are the conditions needed to invoke compensatory responses. First, it takes 4 weeks in hibernation to induce compensation responses in frog motor neurons for breathing. This is shown by a significant increase in synaptic strength following 2 weeks, and maintaining after 4 weeks, showing that the compensatory response saturates. Second, activity needs to be silenced to induce compensation.

Another finding is inactivity induced by hibernation triggers an increase of synaptic strength that are comparable to pharmacological inactivity by TTX. However, synaptic scaling only occurs following hibernation, possible explanations are the longer exposure to inactivity or the environment of cold temperatures. This scaling effect along with compensation persists after hibernation whether neuronal activity is present during the recovery.

Our data also suggests that the scaling effect is actively maintained by calcium-dependent feedback. We draw this conclusion because nimodipine, a selective L-type calcium channel blocker, led to narrower bursts, indicating that scaling independent of compensation acts to regulate outflow to the breathing muscle. Our data strongly suggest that nimodipine is weakening respiratory motor outflow because acute application had no effect on burst width. In addition, nimodipine treatment did not lead to changes in membrane potential (mV), input resistance, firing rate, and mEPSC frequency after hibernation, lending credibility to the idea that it is the scaling organization in and of itself is the main regulator within the network.

The experiments described in this thesis attempted to understand mechanisms of neuron compensation and show that hibernating conditions lead to synaptic scaling, a form of compensation. It is also found that synaptic scaling and compensation can be uncoupled. Which brings up the question, how are the two different? In this project

compensation and synaptic scaling where uncoupled by wintering conditions. It is possible that the long term or more “natural” induction of compensation vs the short term, pharmacological induction of compensation leads to different changes within the neuron, leading to alternative compensation mechanisms.

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