THE UNIVERSITY OF CHICAGO

MUSCARINIC DOWNREGULATION OF SK2-TYPE K^+ CONDUCTANCES PROMOTES INTRINSIC PLASTICITY IN L2/3 PYRAMIDAL NEURONS OF THE MOUSE PRIMARY SOMATOSENSORY CORTEX

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BY

DANIEL FRANKLIN GILL

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List of Abbreviations

ACSF	Artificial Cerebrospinal Fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism Spectrum Disorders
ATP	Adenosine Triphosphate
$CaCl_2$	Calcium Chloride
CCH	Non-hydrolyzable Cholinergic Agonist
ChR2	Channel Rhodopsin-2
CK2	Casein Kinase 2
CO_2	Carbon Dioxide
CR	Conditioned Response
CS	Conditioned Stimulus
CYFIP1	Cytoplasmic FMR1-Interacting Protein 1
CYFIP1	Human gene coding for the CYFIP1 protein
c-Fos	Fos proto-oncogene
eGFP	Enhanced Green Fluorescent Protein
EPSC	Excitatory Post Synaptic Current
EPSP	Excitatory Post Synaptic Potential
GABA	γ-aminobutyric acid
GluR1	Ionotropic Glutamate Receptor Subunit-1
GTP	Guanosine Triphosphate
H89	Selective PKA Inhibitor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP	Intrinsic Plasticity
KCl	Potassium Chloride
KO	Knock Out
KOH	Potassium Hydroxide
LFP	Local Field Potential
LTP	Long-Term Potentiation
LTD	Long-Term Depression
matDp/+	Maternally Duplicated
Mg^{2+}	Magnesium Ion
$MgCl_2$	Magnesium Chloride
mPFC	Median pre-frontal cortex
NaCl	Sodium Chloride
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate Receptor
O_2	Oxygen
OE	Overexpressing
oxo-m	Oxotremorine-m, muscarinic agonist
P	Postnatal Day
patDp/+	Paternally Duplicated
PDS	Paroxysmal Depolarization Shift
PKA	Protein Kinase A
PKC	Protein Kinase C

PP2A	Protein Phosphatase 2A
RT	Room Temperature
S1	Somatosensory Cortex Area 1
SEM	Standard Error of the Mean
SK	Small Conductance Calcium Gated Potassium Channels
SK2	SK channel subtype 2
SK2KO	SK2 Knock-Out
STDP	Spike Timing Dependent Plasticity
TBB	Selective CK2 Inhibitor
tTA	Tetracycline-controlled Transactivator
TRE	Tet Response Element
Ube3a	Ubiquitin-Protein Ligase E3A (murine protein)
UBE3A	Ubiquitin-Protein Ligase E3A (human protein)
UR	Unconditioned Response
WT	Wild-Type

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Thank you to my family for always supporting my odd obsessions. I'll forever be indebted to my siblings who (eventually) helped convince my mom that calling me a "nerd" was a sign of love as well as a compliment, not an insult. This is certainly not the end of putting up with me

rambling on and on about some scientific principle that fascinates me, and your patience and support in professional pursuits and life in general has been crucial for me in completing this thesis.

Technical Abstract

A commonly held understanding in neuroscience is that neurons can adapt their synaptic contacts in order to more efficiently encode information in the context of learning. And while there is no doubt that synaptic encoding is very important for this process, there are additional ways in which neurons can adapt themselves to generate a malleable output in response to incoming stimuli. One such way is to modulate a neurons excitability and shift the entire input-output response curve rather than modulating particular synaptic weights. Muscarinic acetylcholine receptors (mAChRs) inhibit small-conductance calcium activated K^+ channels (SK channels) and can enhance synaptic weight via this mechanism. However, SK channels are also known to be regulated in activity-dependent plasticity of membrane excitability (the cell's 'intrinsic plasticity'). Initially, we investigated whether muscarinic activation can drive SK channel-dependent changes in intrinsic excitability in Layer II/III pyramidal neurons of primary somatosensory cortex (S1). To accomplish this, we utilized whole-cell patch-clamp recordings from these neurons in slice and monitored spiking responses over time to 500 ms depolarizing pulses that initially generate 4-8 spikes before any manipulations. Therefore, if the intrinsic plasticity of the cell were to shift, this could be detected by a decrease or increase in the overall number of spikes during these test pulses. Using this method, we found that brief bath application of the mAChR agonist oxotremorine-m (oxo-m) causes long-term enhancement of excitability in wild-type mice that is not observed in mice deficient of the SK2 isoform (SK2KO mice). It had been shown that mAChR activation could affect several cellular features through SK channels, but had not yet directly shown that it could enhance responses to incoming currents through SK2 channels in particular.

Similarly, repeated injection of depolarizing current pulses (modeled off of Mahon & Charpier 2012) into the soma triggers a shift in intrinsic excitability that is absent from SK2KO mice and thus relies on these channels. When evaluating specifically how spiking phenotypes shift within a single stimulus sweep, we identified that spikes occur more quickly as the cell become more excitable in these conditions. Additionally, we monitored the proportion of spikes that occurred toward the back of an individual stimulus sweep and saw an increase in spiking in the late phase of the test pulses. This lowering of spike attenuation is consistent with SK channel modulation, which can have a 200 ms time constant to become fully activated and shunt off late spiking and produce an after-hyperpolarization.

We next examined what intracellular pathways are responsible for the modulation of SK2 channels, and found depolarization-induced plasticity is prevented by bath application of the protein kinase A (PKA) inhibitor H89, and the casein kinase 2 (CK2) inhibitor TBB, respectively. These findings point toward a recruitment of two known signaling pathways in SK2 regulation: SK channel trafficking (PKA) and reduction of the calcium sensitivity and thus response of SK channels (CK2). Given these results, we also tested the effects of muscarinic activation via oxo-m with H89 in the bath, and found that while PKA was inhibited muscarinic receptors could not induce a change in plasticity. These results offer additional data to the controversial subject of how muscarinic signaling impacts SK channels, and supports two well founded mechanisms by which SK channels can be functionally downregulated. We also utilized mice with an inactivation of CaMKII (T305D mice) which have a mimicked permanent inhibitory phosphorylation of CaMKII. These mice were still able to shift their intrinsic excitability, along with their WT counterparts, even when the induction method required activation of synaptic contacts.

Finally, we demonstrated that repeated injection of depolarizing pulses in the presence of oxo-m causes intrinsic plasticity that surpasses the plasticity amplitude reached by either manipulation alone. This showed that these methods of intrinsic plasticity induction can work together, and we have not hit a ceiling effect in our manipulations. Our findings in total demonstrate that muscarinic activation and increased activation of the soma enhances membrane excitability in layer II/III pyramidal neurons via a downregulation of SK2 channels.

Chapter 1: Introduction and Background on Intrinsic Plasticity

Introduction to Synaptic Plasticity

A central feature of the brain is the ability to encode and store incoming information in order to adapt and change in response to a repeated stimulus. This ability to learn and change is crucial to survival, but is present in nearly every decision made by a complex organism. On the cellular level, the building blocks of these decisions are encoded by neurons. The work of this thesis is largely to better understand the rules that guide these neurons and how they adapt to new incoming information, with a hope that they can offer insight into how neurons encode information more broadly.

Some of the most important cellular 'rules' that underlie learning and memory involve how the synapses between cells encode information. Since its discovery in the 1970's, Long-Term Potentiation (LTP) has been widely viewed as the cellular mechanism underlying learning and memory. LTP is a cellular phenomenon that enables a neuron to selectively strengthen particular inputs onto a neuron without changing the baseline reactivity of that neuron. In 1983, Collingridge et al. made one of the first big leaps in understanding how LTP functioned on a cellular level. Initially, synaptic plasticity was easily studied in the CA3-CA1 synapse in hippocampus due to the ease of electrically stimulating the Shaffer collaterals and reliably causing synchronized release events in CA1. In examining this CA3-CA1 synapse, they discovered that activation of the glutamate-activated NMDA receptor associated channels were crucial in order to potentiate these synapses. They also noted that NMDA receptors did not play a significant role in normal synaptic transmission, but seemed to be only relevant to modulating the strength of the synapses.

In 1984, Mayer et al. and Nowak et al. illustrated how NMDA receptors that could explain this data by offering a mechanism of their function. They discovered that under normal conditions, Mg^{2+} would occupy the channel and not permit any current to flow through the channel. However, if Mg^{2+} was removed from solutions or by depolarizing the post-synaptic cell, other ions were able to flow through the channel into the cell. This meant the channel could act as a 'coincidence detector' that only opened if glutamate was present in the synapse and the cell was depolarized. Importantly, this allowed Ca^{2+} to flow into the post-synaptic cell when the post-synaptic cell was active and the presynaptic terminal released glutamate. Taken together, these results from the early 80's offered the beginning of an explanation for how neurons strengthened their connections. If a pre-synaptic cell was stimulating a post-synaptic neuron and the post-synaptic neuron was sufficiently stimulated, this connection would increase and become potentiated.

LTP is, however, not solely caused by NMDA receptor activation. Various other proteins and receptors, such as metabotropic receptors, will be expanded upon later in this chapter. Regardless, the signaling cascades following NMDA receptor activation continue to be studied to this day. A very important protein following NMDAR activation is Calcium/Calmodulin Dependent Kinase II (CaMKII). CaMKII is associated with the post-synaptic density (PSD), and is capable of responding to local synaptic events and calcium influx. Calcium influx is very tightly controlled in nano-domains within the presynaptic and post-synaptic cell. When calcium enters a cell, the concentration will rise rapidly in the immediate vicinity of the channel and is capable of binding to signaling proteins prior to being rapidly removed. A substantial fraction of Ca^{2+} influx after a synaptic event binds to calmodulin, which can activate various enzymes in order to modify synaptic strength. A major target of calcium/calmodulin is CaMKII, which can

have a myriad of effects on the synapse (Giese & Mizuno 2013). The importance of CaMKII can be demonstrated even on the scale of animal behavior, when mice are subjected to spatial/non-spatial memory tasks.

One such task is the Morris water maze. The Morris water maze consists of a tank of opaque water with a hidden (or visible, for non-spatial tasks) platform. During the test, a mouse or rat is dropped in and timed until it can find the platform and stop swimming. When training rats in this task, their level of training and performance is correlated with CaMKII activity as measured by phosphorylated CaMKII (Tan & Liang 1996). Rats and mice will additionally improve their performance over repeated tests. However, when an inhibitor of CaMKII is injected into the hippocampus prior to testing, rats fail to improve their performance on the task day after day. When this injection took place prior to a non-spatial task (where the platform was visible), they were able to improve their latency to getting on the platform and learn this non-spatial task. There are potential explanations for this discrepancy. The hippocampus is primarily involved in spatial memory tasks, so utilizing visual information could potentially involve separate, non-hippocampal, neural circuits. Another caveat is in this experiment the "non-spatial" group was just 4 rats with no vehicle control group, so their improvement could have been an artifact of probability. Finally, circuits engaged with visual memory may have maintained the memory until inhibitor was no longer present in the hippocampus, at which point the memory was effectively consolidated. These experiments effectively demonstrated the importance of CaMKII, but also illustrated that there are certainly other mechanisms and proteins that are necessary for learning.

From a cellular perspective, the field has generated a robust set of literature showing how NMDA to CaMKII activation can increase synaptic strength. After NMDA receptors are

activated, CaMKII binds calcium/calmodulin and switches from a state that is bound to F-actin more internally in the cell to a state that is bound to the PSD (Shen & Meyer 1999). From there, CaMKII is able to increase synaptic strength by phosphorylating the Ser831 residue on GluR1 to increase its conductance, or CaMKII can recruit additional pathways (such as stargazin) to directly insert more receptors into the membrane (Groc & Choquet 2006, Nicoll et al. 2006). Looking at how CaMKII itself is regulated, this activation is often initiated by Ca^{2+} influx as previously noted but importantly results in autophosphorylation of the Thr286 residue of CaMKII (Lisman et al. 2002). However, other residues such as Thr305/6 can control the activity of CaMKII and prevent or induce it to remain in an inhibited state.

Elgersma et al. in 2002 generated mice to illustrate how this CaMKII 305/6 inhibitory autophosphorylation site functioned. When they substituted the amino acids at 305/6 (naturally threonine) with non-phosphorylatable amino acids (valine and alanine, respectively), they saw an increased affinity for the PSD indicating greater CaMKII activity because it could no longer be inhibited. However, when they substituted Thr305 with negatively charged Asp, the negative charge interferes with calcium/calmodulin binding and effectively mimics permanent inhibitory phosphorylation. This variant of CaMKII didn't associate with the PSD (indicating low activity) which was confirmed by the inability to induce hippocampal LTP. Interestingly, a 10 Hz stimulation protocol for 10 seconds was able to very mildly induce LTD (roughly 90% of baseline), which is noteworthy based on my stimulation protocol that is similar to 10 Hz stimulation. Regardless, the induction of LTP is often dependent on CaMKII and thus classic synaptic plasticity is strongly connected to the function of CaMKII and the ability to increase current through AMPA channels at particular synapses.

A final relevant note on the cellular mechanisms of classical LTP is the concept of spike timing dependent plasticity. The notion as outlined in Markram et al. 1997 is that if an input precedes a spike, that input should be strengthened as it was a useful predictor/driver of the spike. However, if an input follows a spike it is not a useful input in driving post-synaptic activity and ideally should be depressed. And indeed, when trains of input EPSPs preceded post-synaptic spiking, the connection was strengthened (and when EPSPs trailed spiking, the connection depressed). While much work has been done to tease out the pathways responsible for depression, it is notable that the increase in synaptic strength due to STDP can still be explained by classic NMDA leading to calcium/calmodulin and then CaMKII activation to induce LTP.

In fact, classic LTP continues to explain many observations in the brain quite convincingly. A particularly stunning example of tying classical synaptic learning to behavior was shown in Nabavi et al. 2014. In order to demonstrate the importance of synaptic connections in the brain (and show how new tools could exert influence over them in profound ways), they set out to manipulate auditory fear conditioning. Auditory fear conditioning consists of a neutral conditioned stimulus (CS), such as a tone, paired with an aversive unconditioned stimulus (US), such as a foot shock, which results in a conditioned response (CR) whereby the tone is able to drive a fearful response, such as freezing. However, instead of a tone as the CS, they used optogenetic stimulation of neural inputs to the lateral amygdala originating from auditory nuclei. This was accomplished by injecting ChR2 into the medial geniculate nucleus and auditory cortex, then placing a stimulating fiber optic cable into the amygdala to stimulate the ChR2 expressing axon terminals. Using this paradigm, mice learned to associate optogenetic

stimulation with a foot shock. To further illustrate that this learning is dependent on classic LTP, they were able to block this learning from occurring by local diffusion of an NMDA antagonist.

They continued to demonstrate the reliance of fear conditioning on synaptic encoding. Initially, they are able to create a CR as measured by the absence of lever presses during optogenetic stimulation (the CS). However, after they stimulate the afferents at 1 Hz to induce LTD, they are able to extinguish this CR. When they then stimulate at 100 Hz (the ChR2 used is able to follow stimulation up to 100 Hz, where earlier generation variants of ChR2 are not activatable as rapidly) to induce LTP, they recreate the CR and the mice are once again associating the stimulation with a fear response. They continue another cycle, once again eliminating and recreating a CR using LTP and LTD. Looking at this impressive ability to control a fear memory, the importance of synaptic plasticity cannot be doubted. However, outside of modulating synaptic strength, the brain certainly uses other means of encoding information that could occur separately or in parallel. The results of Nabavi et al. might argue that auditory fear conditioning is dependent solely on synaptic strength, and it is certainly noteworthy that this learning doesn't require neuron outgrowth to form novel connections, but instead is merely activating/inactivating synapses. However, some forms of learning cannot be explained by solely modulating synaptic strength. This method of encoding 'intrinsic excitability' involves shifting its response to all inputs globally, rather than selectively as in synaptic plasticity.

Introduction and Overview of Intrinsic Plasticity

As overviewed in the previous section, synaptic plasticity is clearly of importance in encoding and accessing information relevant to learning. However, neurons have additional ways of encoding information that could be more effective for certain tasks, such as shifting the

excitability of the entire cell. One such 'task' is grouping together neurons to form an ensemble code which will be explored in this chapter. As outlined in Figure 1 adapted from Titley et al. 2017, the concept of a 'neuro-centric' view of learning includes synaptic plasticity among many possible things that can shift a neuron's response to information.

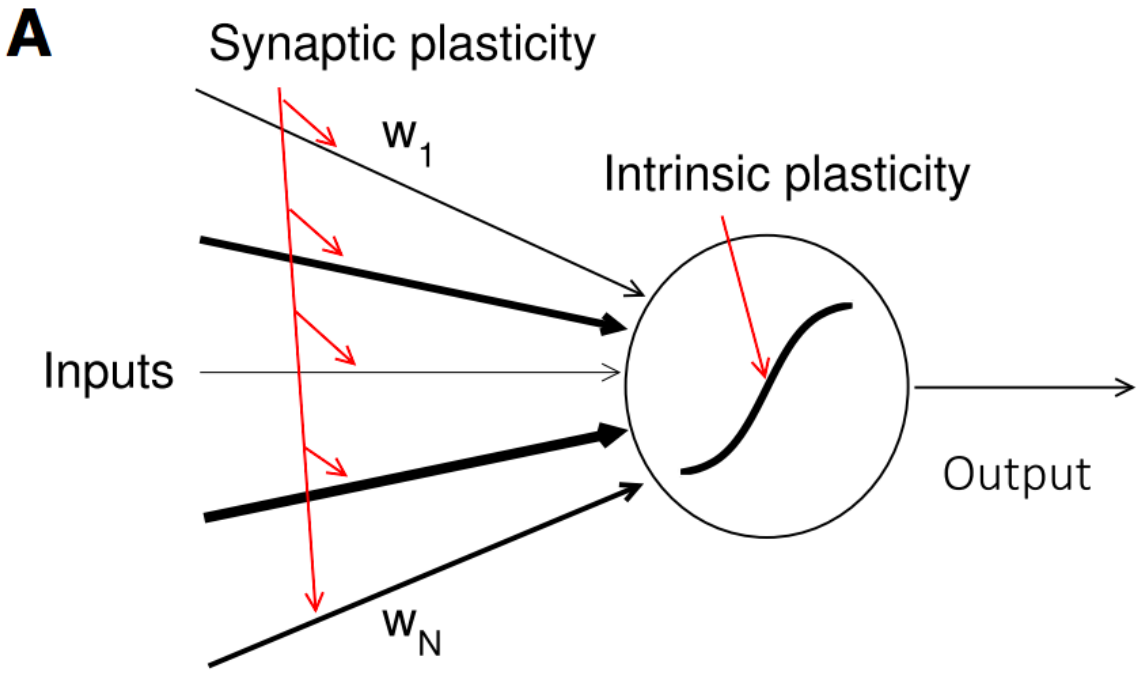

Figure 1: "Neuro-centric" View of Learning. In this view of cellular encoding of information, synaptic plasticity has the ability to shift weights of individual incoming neurons. However, the post-synaptic cell can also shift its response to all inputs by modulating its excitability. Adapted from Titley et al. 2017.

This model of encoding memories allows for explanation of a larger range of phenomenon than synaptic plasticity alone. In particular, it might explain neuronal 'engrams' or groups of neurons that when activated together can encode a memory. To begin examining the role of intrinsic excitability, it is useful to examine a concrete example. Such an example can also be found through fear conditioning, although in this case the association will not be with a

tone but instead a particular context (location). The Tonegawa lab developed a method of creating and stimulating engrams that has proven very useful in understanding how these types of memories are encoded. To begin, they generated a mouse that expresses ChR2 that is both time and activity locked. In Liu et al. 2014, ChR2 was expressed under a TRE (Tetracycline Responsive Element) promotor that relied on the absence of doxycycline so tTA could bind it, and tTA was driven by c-Fos so was thus activity dependent. This setup is incredibly useful because it allows researchers to keep animals on tetracycline/doxycycline and not allow ChR2 expression. However, during specific periods (such as exposure to a novel environment), tetra/doxycycline can be briefly stopped and the cells that are active during this period will express ChR2. This expression then allows independent activation at a later time of the network of neurons that expressed c-Fos and now express ChR2.

To illustrate how an engram can be encoded, Liu et al. 2014 first exposed mice to a context "A" while on doxycycline. Following this, the mice were removed from doxycycline and fear-conditioned in context "B", learning to associate context B with the threat of a foot shock. This also allowed hippocampal neurons which were active in context B to express ChR2. Once back on doxycycline, mice were replaced into context A and monitored for fear responses. As expected, the mice were not fearful in context A, as they were treated fine in this context and not shocked like in context B. However, when the group of neurons expressing ChR2 (engram encoding context B) was activated in context A, the mice began to freeze and show signs of a fear association. This association could also be stopped by turning off the light, and re-engaged by turning the light back on. This result encapsulates the concept of an engram, whereby a group of neuron's collective co-activity effectively encodes a memory.

One possible explanation of this result is that this engram is encoded through these neurons changing their intrinsic excitability. Interpreting this result under normal conditions, a certain context is encoded by shifting the excitability of all neurons in the engram such that they are more sensitive to all inputs and thus fire more frequently. In these ChR2 mice, a context is made 'active' by stimulating these neurons and having them fire frequently (it is also worth noting that this stimulation is very broad somatic stimulation and not at all tied to particular synaptic inputs). When the mice are placed in context A and activated, this non-specific somatic stimulation would reactivate the engram and could reactivate associated fear-encoding pathways. While this is a plausible explanation, the experimental result by itself doesn't require an explanation other than classic LTP/LTD. It is very possible that these neurons became grouped together using classic synaptic plasticity and the idea of an engram is just secondary to a synaptic network.

To further examine the idea that this engram is a consequence of LTP/LTD, this would mean that these neurons become linked synaptically through classic plasticity. Therefore, this non-specific activation would just be activating the synaptic links that have been forged and driving a fear response. However, when this experiment was repeated with an injection of anisomycin (which blocks protein translation, thus blocking the necessary steps of LTP) or saline following the initial fear conditioning, intrinsic plasticity becomes a compelling explanation. After fear conditioning in context B (and receiving an injection), mice were placed back in context B the following day and monitored for a fear response (which should be present). Mice with saline injected understandably showed an increase in freezing indicating they had effectively associated fear with context B (Ryan et al. 2015). This rate of freezing was nearly 3x higher than freezing in mice injected with anisomycin, indicating that the anisomycin injected

mice had not effectively encoded/recalled this fear association. When examining cellular features, hippocampal neurons from the saline mice had undergone LTP and seen an increase in spine density that was not present in the anisomycin group, indicating that LTP had been eliminated in this group. It is perhaps not shocking that inhibiting LTP caused the loss of a context dependent memory, but the following day the ChR2 expressing mice were placed back into the neutral context A. Initially they didn't show a fear response; however, when the context B engram was stimulated, both the saline and anisomycin group demonstrated a clear fear association (not present in those who didn't receive a foot shock). Reactivating this engram in the anisomycin group, which had not been encoded synaptically, allowed for reinstating of the fear association. This leads to the conclusion that the engram is somehow driving connectivity, not the other way around. Intrinsic plasticity offers a robust explanation for how a neuron could shift its excitability to encode being part of an engram, without requiring synaptic plasticity. There is likely overlap of IP with LTP and shifting excitability alongside growing synaptic connections can possibly more fully express a memory. More recently in Pignatelli et al. 2019, the impact of cellular excitability on hippocampal engram formation was explored. They discovered that briefly, for less than 2 hours following fear conditioning, a transient increase in membrane resistance and reduction in rheobase occurred in cells incorporated into the engram. These particular changes were due to transient downregulation of inward-rectifying potassium channels, and artificially inserting these channels into engram cells disrupted behavioral performance. While these results demonstrate the role that these channels play, other metrics of excitability are not explored (such as response to sustained current injection) that could assist in consolidation of the memory. In particular, Calcium-activated potassium channels have a

particular rationale that will be explored later in this chapter, and the concept of overlap between LTP and intrinsic plasticity will be explored more fully in Chapter 3.

From a cellular perspective, intrinsic plasticity can be used to effectively encode information. Intrinsic excitability alone has been shown to encode differential responses to identical inputs (i.e. a cellular 'memory'), allowing a cell to transition from spiking to tonic firing that is dependent on intrinsic plasticity (Marder et al. 1996). This shift is noteworthy because initially a cell with a 'spiking' phenotype might just generate a short, single burst of synaptic release in response to sustained input. When shifting to tonic, the synaptic release can continue as long as inputs to the cell are active, offering a differential and more influential response given the same input. This is not to say that intrinsic plasticity entirely replaces LTP, but can serve a complementary role in general plasticity independent of adjusting individual synaptic weights. For example, changes in intrinsic excitability were able to effectively couple incoming EPSPs to postsynaptic spiking without altering synaptic plasticity (Sourdet et al. 2003). Additionally, when neurons have their intrinsic excitability increased, it has been shown that they are more likely to synchronize their individual activity, offering further cellular support for intrinsic plasticity as a mechanism of engram formation (Rodriguez et al. 2004; Betterton et al. 2017). The continued conceptual overlap of synaptic and intrinsic plasticity will be explored in the context of our results in chapter 3.

In our research we chose to examine pyramidal neurons of cortex, which in particular have been shown to modulate their excitability in response to differential inputs. Additionally, engrams have been found in cortex to occur through relevant physiological stimulations (i.e. engrams in visual cortex develop when a mouse is exposed to visual stimuli), although engrams can also be induced through direct somatic stimulation (Carrillo-Reed et al. 2016). Looking more

specifically, cortex is a layered structure that can be modeled as in figure 2, adapted from Douglas & Martin 2004. Although there is variance between cortical areas, a common feature is incoming synapses often terminate in layer II/III, making it a main input layer. Layer V neurons often project to different cortical regions, making it a common output layer. Neurons in both layers II/III and V have been shown to modulate their intrinsic excitability.

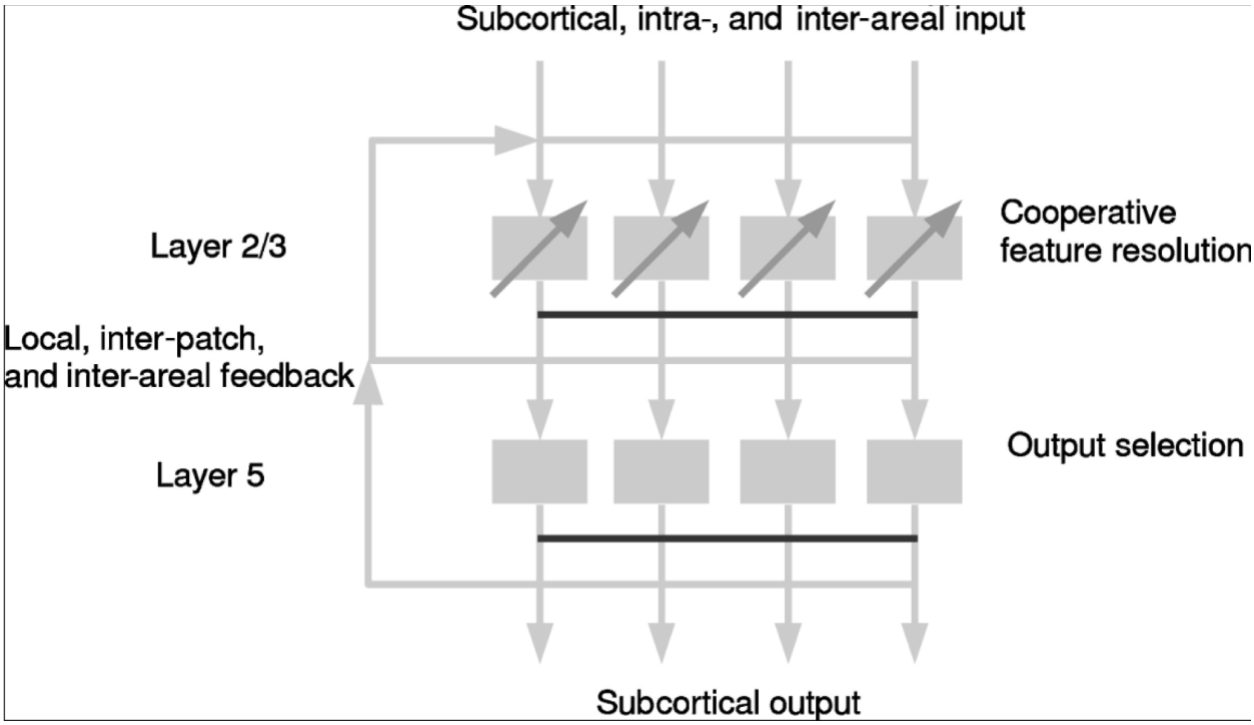

Figure 2: Model of Cortical Circuits. This schematic summarizes many of the findings about cortical micro-circuitry within a particular cortical column. Inputs flow into layer II/III and then after undergoing local micro-circuits will eventually flow out of a particular area of cortex through layer V pyramidal neurons. (Adapted from Figure 6 in Douglas & Martin 2004).

In layer V of mPFC, a subset of pyramidal neurons was shown to reduce their intrinsic excitability in response to social isolation of the mice (Yamamuro et al. 2018). In rats learning an operant task, layer V pyramidal neurons of mPFC have shown to increase their excitability when incorporated into an ensemble and decrease their excitability over time when excluded from an ensemble (Whitaker et al. 2017). In layer II/III, some of the observed results have offered a dynamic view of intrinsic plasticity over time. In primary motor cortex of rats, layer II/III

neurons initially showed lower intrinsic excitability after the first day of learning a new task. However, this trend reversed on the second day, where neurons showed a robust increase in intrinsic excitability (Kida et al. 2016). Looking more specifically at layer II/III pyramidal neurons of barrel cortex in mice, dynamic responses can emerge with an ultimate potentiation in response to whisker trimming and stimulation (Glazewski et al. 2017). When principal whisker responses were recorded, the response of layer II/III neurons initially depresses from deprivation, but ultimately potentiates to roughly 150% of baseline.

To better understand and characterize the changes in intrinsic excitability of layer II/III pyramidal neurons of somatosensory cortex (S1), we sought to identify an electrophysiological protocol that can induce such changes. In Mahon & Charpier 2012, a protocol is identified that is capable of bidirectionally modifying the excitability of layer V pyramidal neurons of barrel cortex in rats (based upon existing cellular features). We sought to adapt their method of inducing changes in excitability to layer II/III pyramidal neurons in mice, in order to begin teasing out some of the mechanisms underlying this effect. The specifics of their methods and our results will be examined further in chapter 2, but now I will provide further background on some of those mechanisms we hope to tease out.

Muscarinic Signaling Background

Muscarinic Acetylcholine receptors are G-Protein Coupled Receptors (GPCRs) that bind Acetylcholine, and a wide variety of cellular responses can be elicited through activation of the associated subunits. In the particular case of muscarinic receptors, there are five subtypes of the receptors which can activate different G-protein pathways (Kruse et al. 2014). Of the 5 subtypes of muscarinic GPCRs (numbered M_1 through M_5), M_1, M_3, and M_5 have been shown to couple to the G_q family activating PLC and later PKC/PKA (Chen et al. 2017), while M_2 and M_4 couple to

the $G_{i/o}$ family, inhibiting cAMP production in opposition to the G_s family (Hulme et al. 1990). A summary of these pathways can be seen in figure 3, adapted from Wess et al. 2007.

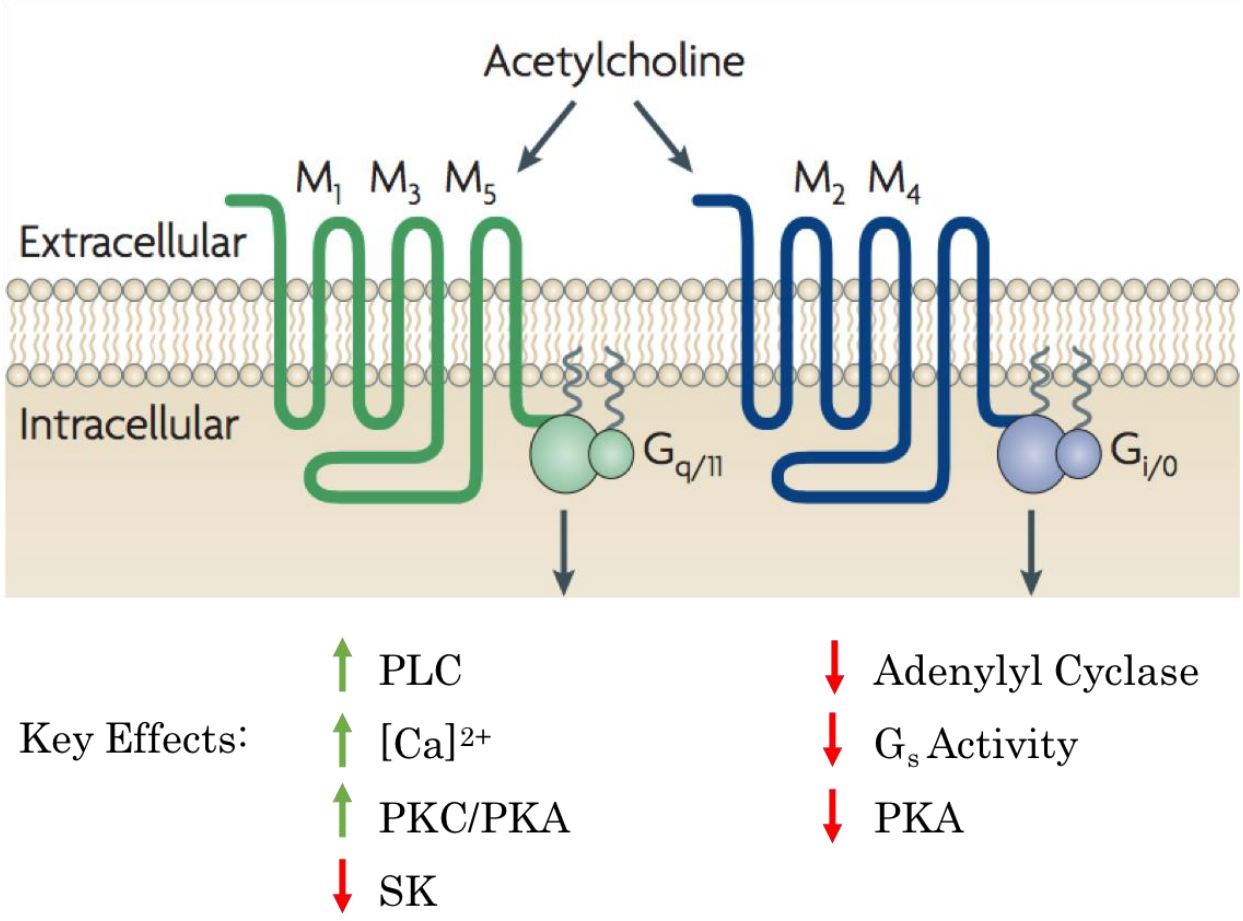

Figure 3: Muscarinic Signaling Pathways. This summarizes the divergent pathways through which muscarinic signaling occurs. (Adapted from Wess et al. 2007)

Muscarinic receptors have shown to have an important role in learning and cellular plasticity in particular. In 1986, Bear & Singer showed the importance of cholinergic signaling on visual plasticity by measuring ocular dominance in kittens while disrupting cholinergic (and adrenergic) signaling. While this early work did not isolate muscarinic signaling, Kirkwood et al. in 1999 showed that synaptic LTD could be eliminated in the visual cortex via an M_1 antagonist. This was further refined in 2006 to show that in visual cortex M_1 and M_3 were required for LTD,

while M_2 and M_4 were required for LTP (Origlia et al. 2006). While these results have offered a clean distinction between muscarinic receptors in synaptic plasticity, it is also worth noting that the when it comes to intra-cellular signaling cascades, muscarinic receptors create a variety of downstream effects. After activating the relevant G-protein subunits, the end effects and targets of these messengers are still being studied. As an example of that complexity, it was later revealed that the ultimate mechanism of M_1/M_3 dependent LTD in the visual cortex relied on endocannabinoid signaling, indicating that the downstream targets of these pathways are often difficult to tease out (Zhao & Tzounopoulos 2011).

Outside of synaptic plasticity, muscarinic receptors were of particular interest to this project because of their documented ability to shift cellular excitability. In hippocampal CA1 neurons, activation of muscarinic receptors is able to induce a change in excitability largely caused by the elimination of an after-hyperpolarizing current following burst firing (Park & Spruston 2012). This increase can be caused by generic cholinergic activation using carbachol (abbreviated CCH, which would hit ionotropic/nicotinic receptors as well), but is mirrored by application of a specific muscarinic agonist, oxo-m. Application of a specific M_1 agonist also increased excitability, and the CCH dependent increase was eliminated with an M_1 specific (and M_1/M_3 specific) antagonist. The observed increase was unaffected blocking the even-receptors, indicating that the odd-receptors (and M_1 in particular) were responsible for this increase in excitability. Further experiments demonstrated the cholinergic based increase in excitability of CA3 neurons necessary for cellular activity-synchronization is reliant on M_1 activation (Betterton et al. 2017), offering additional support for the odd-receptors driving important changes in excitability.

In layer V cortical neurons, after-hyperpolarizing currents are mediated by muscarinic receptors, and these currents can be reduced in order to increase cellular excitability (Joshi et al. 2016). Layer II/III and V neurons of rat infralimbic cortex likewise increase their response to depolarizing current when treated with muscarine (Santini et al. 2012). While this result is not subtype specific to M_1 and M_3 like in hippocampus, given the previous results these are likely consistent with G_q activation via the odd-receptor family. Like uncovering the pathways and targets downstream of muscarinic activation in LTP/LTD, it is of interest to this project to understand how muscarinic signaling might impact intrinsic excitability. One potential target of interest is SK channels, which will be introduced in the following section and then tied into the body of knowledge about their interactions with muscarinic receptors.

SK Channels Overview

SK channels are a group of small-conductance calcium-activated potassium channels, which underlie the after-hyperpolarization of neurons following activity (and thus calcium entry) (Stocker et al. 1999). Specifically, these channels underlie the medium-fast component of after-hyperpolarization (mAHP) with a time constant of roughly 200 ms (Villalobos et al. 2004). This identifies SK channels as a candidate for influencing neuronal intrinsic excitability because sustained firing becomes more difficult as SK channels allow a hyperpolarizing efflux of potassium. This theory has been borne out as application of apamin, a specific SK antagonist, can occlude increases in intrinsic excitability of layer V pyramidal neurons (Sourdet et al. 2003). Additionally, SK channels have the potential to influence many potential brain areas. All three subtypes of SK channels are expressed in cortex at varying levels, and at least one subtype is expressed in nearly every brain region (Gymnopoulus et al. 2013). With regards to narrowing down to a particular subtype of interest in layer II/III of cortex, SK2 channels have been shown

to be necessary for intrinsic plasticity of Purkinje cells, although this particular result might be due to SK2 being the primary SK expressed in cerebellum (Grasselli et al. 2016). However, using KO mouse studies, it was determined that SK2 is the only SK necessary for after-hyperpolarizing currents blocked by apamin, a selective SK antagonist, in hippocampal CA1 neurons (Bond et al. 2004). Additionally, overexpressing SK2 channels (approximately 10x WT) increased this apamin sensitive current 4x, and EPSP magnitude increased roughly 2x following apamin block (Hammond et al. 2006). Given these results, we sought to further investigate SK2 channels in the context of intrinsic plasticity in layer II/III pyramidal neurons.

Because we will utilize cells from mice with an SK2 KO, it is worthwhile to discuss the known behavioral effects of SK channel modulation. The mice we test were initially generated in Bond et al. 2004, and their generation involved replacing exons 1&2 of *SK2* with a non-functional eGFP. It is worth mentioning that the non-functionality of eGFP is currently being studied as part of a future direction of this project that will be mentioned in Chapter 3. Regardless, SK2 is effectively removed from the genome of these mice and there are behavioral consequences. The visual appearance of these mice is 'shaky' as they cannot properly coordinate their movements and a very clear tremor is noticeable which is somewhat similar to human ataxia. For this reason, these mice haven't been thoroughly categorized for learning/memory deficits, as their 'ataxic' phenotype would certainly interfere with interpretation of experimental results. However, we can perhaps get an indirect assessment of an SK2KO by looking at the research conducted on mice treated with apamin and studies conducted on transgenic mice over-expressing SK channels.

The closest model of SK2KO mice could be mice injected with apamin, so that the SK channels would be inactivated. Interestingly, mice injected with apamin were able to more

quickly learn a bar pressing task and exhibited an increase in overall behavioral activity, which included pressing the bar more frequently (Messier et al. 1991). When tested on a larger array of behavioral tests (due to apamin's potential as a cognitive enhancer), results were mixed. Mice injected with apamin trended towards faster acquisition in the Morris water maze, but this result was not significant, although the treated mice spent more time in the trained quadrant (van der Staay et al. 1999). Overall, rats did not show clear cognitive enhancement when treated with apamin (including in a lever pressing task), and although mice had some positive results of enhancement, the confounding variable of increasing activity (often seen as reduced immobility) couldn't be eliminated. For that reason, the researchers concluded in their discussion that apamin was not a useful tool in studying SK channels in regards to behavior, which does leave a gap in knowledge for the intellectual deficits/gains in SK2KO mice. Additionally, the maximum dosage used in testing for mice was 0.3 mg/kg. However, when tested in rats, only a dosage of 1.0 mg/kg was able to induce a tremor which would be a phenotype more closely aligned with SK2KO mice (although these rats had seizures as well, which has not been observed in these mice). In conclusion, apamin treatment is perhaps not suitable for modeling and better characterizing SK2KO mice, but the possibility that these mice have some cognitive gains/deficits cannot be completely ruled out, and introduce the potential for confounding variables. While our studies are slice and not behavioral, cognitive differences could potentially influence neuronal function and alter it impacting our comparisons to WT mice cells.

Other than reducing SK activity, experiments have been conducted on mice which overexpress SK2. In the Morris water maze, these mice were capable of locating and swimming to a visible platform, but had deficits in recalling the quadrant in which the platform was located (Hammond et al. 2006). When tested during fear conditioning, overexpressing mice also showed

a reduction in the association between a context, tone, and foot shock (Stackman et al. 2008). This is of particular interest given the connection between hippocampal engrams and intrinsic plasticity that was previously discussed. Finally, focal application of NS309 in the hippocampus, a pharmacological SK channel enhancer, impaired trace eyeblink conditioning (using a 250 ms trace interval between tone and air puff) in rats (McKay et al. 2012). This effectively increased SK activity, and due to focal application and previous studies implicating SK2 in the hippocampus, the assumption is SK2 is primarily affected. Additionally, after trace learning SK2 mRNA levels were measured and were shown to be reduced.

Trying to reconcile these apamin and overexpression/overactivity experiments, it is possible when viewing potential flaws in the data favorably to draw a correlation that more SK leads to poor memory and cognition, while less SK leads to improved memory and cognition. As stated before, the relevance of these apamin experiments on SK2KO mice is questioned even by these researchers. Nevertheless, it is possible that if this correlation is true, it would imply these SK2KO mice have significant cognitive advantages. This is unlikely, given that ensuring the survival of these KO mice in colonies requires thinning of the litter to a manageable size to reduce competition for milk etc., and later requires gel feeding. A more likely interpretation is that the apamin results drove an increase in overall activity through some side-effect of administration, and this increase in activity correlated with an increase in operant conditioning tasks. A more plausible interpretation SK2 is calibrated to a particular level and significant departures in expression are likely to have negative behavioral consequences. This could explain learning deficits in overexpressing mice, and would also imply that the SK2KO have learning deficits (and chapter 2 will discuss how these mice have deficits in intrinsic plasticity). Perhaps a

test could be contrived that will effectively control their ataxia in order to properly test their cognition, but as of now any direct behavioral results on SK2KO mice are lacking.

Eschewing behavior for cellular functionality, it is of interest to understand how SK channels can be downregulated mechanistically. Ion channel function can be broken down into three possible pathways of regulation. First and foremost, the dependence of an ion channel on its activating partner could be shifted such that it no longer is activated as easily. Second, the conductance of the channel could be altered (or in an extreme case, eliminated due to antagonism) such that current is able to flow easily/difficultly or for a different period of time. Lastly, the channel can be trafficked out of the membrane and/or degraded. Two of the main regulatory pathways that decrease SK2 functionality are PKA and CK2. PKA is specifically involved in internalization of the SK2 channel, where inhibiting PKA or infusing a cell with a peptide that blocks PKA phosphorylation sites on SK2 prevents removal from the membrane (Lin et al. 2008). On the other hand, CK2 is capable of reducing SK channel sensitivity to calcium (Allen et al. 2007). This is accomplished because SK channels are activated by calcium through Calmodulin binding one of four pores in the assembled SK channel. CK2 binds to SK channels and is capable of phosphorylating Calmodulin and reduce its binding affinity to SK, preventing it from being activated. Additionally, PP2A is bound to SK channels and will dephosphorylate Calmodulin. Usually these two proteins work in concert and balance one another out, setting the binding affinity of Calmodulin to an equilibrated level. It is worth mentioning, as later experiments will pharmacologically interact with CK2, that inhibiting CK2 is potentially disruptive for SK channel function. Because the exact binding arrangement is not known for the entirety of the SK2/CK2/PP2A/Calmodulin complex, inhibiting CK2 could allow PP2A to dominate removal of phosphate from Calmodulin and severely shift the sensitivity of

SK channels, resulting in them consistently being open. However, depending on the inhibition, CK2 may remain bound to the channel while inhibited, and this added molecular player could interfere with additional physical channel interaction (such as PKA phosphorylating the channel to tag for removal). This complex protein interaction alongside drug experiments will be referenced later in the context of our results, however CK2/PP2A interaction is a difficult story to untangle.

Lastly, I'd like to explore the interaction between SK channels and muscarinic signaling. As mentioned before, muscarinic receptors merely trigger a G_q or $G_{i/o}$ cascade that can impact SK channels further down the pathway, and the specific interactions are sometimes not understood. However, there have been experiments that have tied muscarinic activation to an effect on SK channels, so it is worth delving into the results in this topic. In 2000, Fiorillo & Williams demonstrated in ventral midbrain dopamine neurons that muscarine wash-on in the bath could reduce the after-hyperpolarization evoked by ACh stimulation (hitting nicotinic receptors and fully activating muscarinic receptors) that corresponded with a decreased calcium conductance, implicating calcium-activated potassium channels as a downstream target of muscarinic receptors.

This result is merely a first step in understanding the signaling cascades, however it is also complicated by the fact that muscarinic activation seems to have an immediate and transient activation of SK channels (Gulledge & Stuart 2005). Slow, metabotropic-linked currents following ACh stimulation induce a hyperpolarization of layer V pyramidal neurons that can be blocked with apamin and is thus SK dependent. As previously outlined, the odd-type muscarinic receptors will activate the G_q pathway which will, through PLC, liberate internal stores of calcium and eventually activate PKC and PKA (Chen et al. 2017). PKA is fully capable of

internalizing SK channels, however this process requires PKA tagging and subsequent removal. In the meantime, calcium will activate SK channels causing a hyperpolarization. This temporary hyperpolarization may well serve an important function, but in the long run if the signaling cascade is complete (due to all necessary cellular factors being present), it may well lead to an elimination of this very effect.

On a short-term basis during large activation, neurons may have to more effectively screen incoming synaptic contacts. On a long-term basis, those that are sufficiently stimulated to internalize SK channels may become grouped as part of an engram code and become more correlated in their activity. This is potentially related but somewhat different from the observations that general cholinergic release can increase signal to noise by increasing excitability (Cohen and Maunsell 2009). Their result involves all cholinergic activation increasing gain and due to the distribution of firing rates creating a greater disparity between a preferred and non-preferred stimulus. However, this tuning is on a short time scale within a single attentional trial. General SK downregulation could impact this tuning by raising the response rate of all neurons throughout all trials.

In back to back studies published by Buchanan et al. 2010 and Giessel & Sabatini 2010 in adjoining pages in Neuron, an attempt is made to sort out the mechanism by which muscarinic receptors cause a reduction in SK current. Buchanan et al. shows that activation of M_1 receptors in hippocampus enhance NMDA-R currents by decreasing SK channel currents. They initially show that this NMDA-R current is extended by application of apamin, and that this effect is unchanged by application of an M_1 agonist, indicating that the extension had reached a ceiling effect and the agonist action was occluded. Examining the after-hyperpolarization current, they find that agonist treatment unsurprisingly reduces this current as SK channels are eliminated.

However, when a PKC inhibitor is used alongside the M_1 agonist, the current is not eliminated to the same magnitude, indicating that PKC was an important step in reducing the SK current. Additionally, inhibiting CK2 (using TBB) did not reduce the reduction in after-hyperpolarizing current. This result indicated that CK2 was not necessary to reduce the SK current changes caused my M_1 activation. Finally, they saw that TBB was able to extend the NMDA-R current, with a magnitude that was even greater than apamin. CK2 can directly impact NMDA-Rs, so this was perhaps not surprising. When they saw an extension that was even greater than apamin, they attempted to draw conclusions about SK channels directly. Given the direct impact of CK2 on NMDA-Rs though, it seems bold to try and divine information about a secondary pathway through muscarinic activation when a primary target pathway is active, so this result should likely be taken with some large caveats. Regardless, in isolating the after-hyperpolarizing current, they offered evidence that reduction of the current relied on PKC activation (which may be a necessary step in activating PKA—Chen et al. 2017) and did not rely on CK2 activation.

On the previous pages of the journal, Giessel & Sabatini showed that general muscarinic activation was able to increase excitability through the functional elimination of SK channels. However, their results point towards CK2 as an important mediating step in the reduction. They are able to show that oxo-m activation of muscarinic channels shifts the after-hyperpolarizing current's sensitivity to calcium using optical Ca^{2+} uncaging, implicating CK2. To attempt to directly implicate CK2, they show that pre-treatment with TBB eliminates any effect from oxo-m wash on from impacting uEPSPs (calcium uncaging evoked EPSPs) and calcium transients, arguing that CK2 is necessary for any effect. Unfortunately, TBB has an impact on NMDA receptors which have a high likelihood of impacting uEPSP measurements. Therefore, I can only

conclude that the evidence for CK2 implication is a shift in calcium sensitivity. They did not examine the role of PKC/PKA.

Looking at the evidence from Buchanan et al., it is easy to conclude that PKC was important for the reduction in after-hyperpolarizing current. However, they used TBB to measure the importance of CK2, showing that it does not occlude the reduction in after-hyperpolarizing current seen in M_1 activation. This can mean CK2 is unimportant in modifying SK channel function. There are certainly issues with concluding too much from small molecule inhibitor studies. The physical environment of the SK channel has a myriad of bound proteins, and access to any particular protein (such as CK2) could be impeded, impacting the function of an inhibitor. In the particular case of TBB, functional elimination of CK2 would be by definition fully activating PP2A with unclear consequences. While it is possible CK2 truly doesn't have role here, and SK2 is solely reliant on the PKC (and PKA) pathway, more data is needed to achieve more confidence surrounding the role of CK2.

This is further complicated by Giessel & Sabatini, who measured the calcium sensitivity of the channel shifting after muscarinic activation, which strongly implicates CK2 as a mediator. However, it is possible that their light-mediated uncaging method of measuring sensitivity could be interpreted differently. By causing smaller or larger clusters of calcium to become free, these results could simply be a measure of receptor density (and thus a measure of internalization, implicating PKA). Ultimately, these two papers come to different conclusions and the pathways are complex and still being teased apart. Or it is possible that CK2 is M_3 or M_5 dependent in some unknown way, which would not have been seen in M_1 activation (and no involvement of PKC was tested for there).

Chapter 2: Expression and Mechanisms of Intrinsic Plasticity of Layer II/III Pyramidal Neurons of Somatosensory Cortex

Materials and Methods

Pyramidal cell recordings ex vivo: Thalamocortical slices (350 μm) were prepared (orientation described in Agmom & Connors, 1991) from postnatal day P25-40 mice after isoflurane anesthesia and decapitation. This procedure is in accordance with the guidelines of the Animal Care and Use Committee of the University of Chicago. The slices were cut on a vibratome (Leica VT1000S) using ceramic blades. The slices were cut in a sucrose slicing solution containing the following (in mM): 185 Sucrose, 2.5 KCl, 25 Glucose, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, and $MgCl_2$, bubbled with 95% O_2 and 5% CO_2. Following slicing, the slices were kept in artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose, bubbled with 95% O_2 and 5% CO_2. The slices were allowed to recover for at least 1 h, and were then transferred to a submerged recording chamber superfused with ACSF at near-physiological temperature (31-34°C). This was an attempt to make the recording conditions more closely physiological, balanced with a need to maintain stable patches after manipulating a cell for a minimum of 40 minutes but often times a full hour (after patching and stabilizing initially). Whole-cell patch-clamp recordings were performed under visual control using a 40X water-immersion objective mounted on a Zeiss Axioskop 2FS microscope. Patch pipettes (2.5-4.5 MΩ) were filled with internal saline containing the following (in mM): 9 KCl, 10 KOH, 120 K-gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 Na_2ATP, 0.4 Na_3GTP, and 17.5 sucrose, pH adjusted to 7.25. TBB (CK2 inhibitor) and oxotremorine-m (muscarinic agonist) were purchased from Tocris. H89 (PKA inhibitor) was purchased from Abcam. Patch-clamp recordings were performed in current-clamp

mode (capacitance cancellation switched off) using an EPC-10 amplifier (HEKA Electronics). Membrane voltage and current were filtered at 3 kHz, digitized at 10 kHz, and acquired using Pulse software (HEKA Electronics). After whole-cell patching in voltage clamp, series resistance was measured and bridge compensated once in current clamp. Before recording in current clamp, a bias current was applied to prevent spontaneous spike activity and to set baseline voltage to -70 mV. Intrinsic plasticity was monitored during test periods by injection of brief (500 ms) depolarizing current pulses adjusted to evoke 4-8 spikes. The spike count was taken as a measure of excitability. Input resistance (R_i) was measured by injection of hyperpolarizing test currents (100 pA, 100 ms) and was calculated from the voltage transient toward the end of current injection. This resistance was monitored and will be commented upon later, but did not have an impact on our experiments. We are counting spikes during a depolarizing pulse, which does not rely on fine-accuracy voltage sensing. When the voltage drops over series resistance and then again over the cell membrane resistor/capacitor combination in between the patching and bath electrodes, the current in this circuit remains stable regardless of series resistance. Since we are initially compensating for the voltage drop across series resistance, we have an initial accurate measurement of membrane resistance. Changes in series can be large (so long as they are not so large that access to the cell is corrupted) without affecting the cell. Series often changes during recording, so this is a useful in our experimental setup. However, membrane resistance can also change during the experiment and this might lead to an increase in excitability. Because we initially compensate for series (and remain in current clamp so cannot directly test for series resistance), observed changes in resistance might come from the cell or from changes in series (that will be amplified in magnitude because the cell is initially compensated from the baseline value). Once an experiment is complete, if appropriate series would be tested again and any

changes noted that might impact analysis (as the series compensation also shifts perceived voltage across the cell as series changes). During recordings the cell is in current clamp, so series is not tested throughout the duration of the experiment and is just a single measure at the beginning and end.

Genetically modified mice: SK2KO mice were originally described in Bond et al., 2004. They were generated on the C57Bl/6 background (mice were obtained from John P. Adelman, OHSU). T305D mice were originally described in Elgersma et al., 2002 (mice were obtained from Y. Elgersma, Erasmus MC, Rotterdam, The Netherlands), and were likewise generated on the C57Bl/6 background. All mice that were not genetically modified were C57Bl/6 mice.

Data analysis: Data obtained from the pyramidal cell recordings *ex vivo* were analyzed using Pulsefit (HEKA Electronics), Igor Pro software (WaveMetrics) and R. For statistical analysis, we used the paired Student's *t*-test and the Mann—Whitney *U* test, when appropriate. Baseline periods were an average of 5 minutes prior to stimulations, and post periods were calculated in each group as an average of the relevant measurements 23-27 minutes following stimulation. This window was initially chosen because not all cells survive the full 30 minutes, and might only make it to minute 25 of post before a strong gust of air conditioning disrupts the cell or another graduate student might accidentally bump the air table. The window was thus shifted in order to allow analysis of some of the cells that otherwise fit the criteria but happened to die early. Within group measures compared the difference between an individual cell's baseline and post, using a paired Student's *t*-test. Between group measurements of more than 2 groups used the Kruskal—Wallis test, and Mann—Whitney *U* tests were used to directly compare groups. In all figures, the values shown represent the mean ± SEM.

Muscarinic Signaling Occurs through SK2 Channels

To monitor intrinsic excitability of Layer II/III pyramidal neurons, we injected brief depolarizing currents (500 ms) that were adjusted to evoke 4-8 spikes as a stable baseline measure of excitability. Under control conditions, where no drug was applied or electrical stimulation applied, we showed that excitability was stable over the entire 40-minute recording session (103±8% of baseline, n=7, p=0.68, Figure 4). We then sought to monitor the changes observed in the presence of muscarinic activation. This was accomplished by bath application of the muscarinic agonist oxotremorine-m (oxo-m) at 7 μM for 5 minutes. It should be noted that for the control ACSF conditions, ACSF was washed on from a separate flask (similar to how oxo-m was washed on) in order to account for any unexpected variables and changes due to switching the bath.

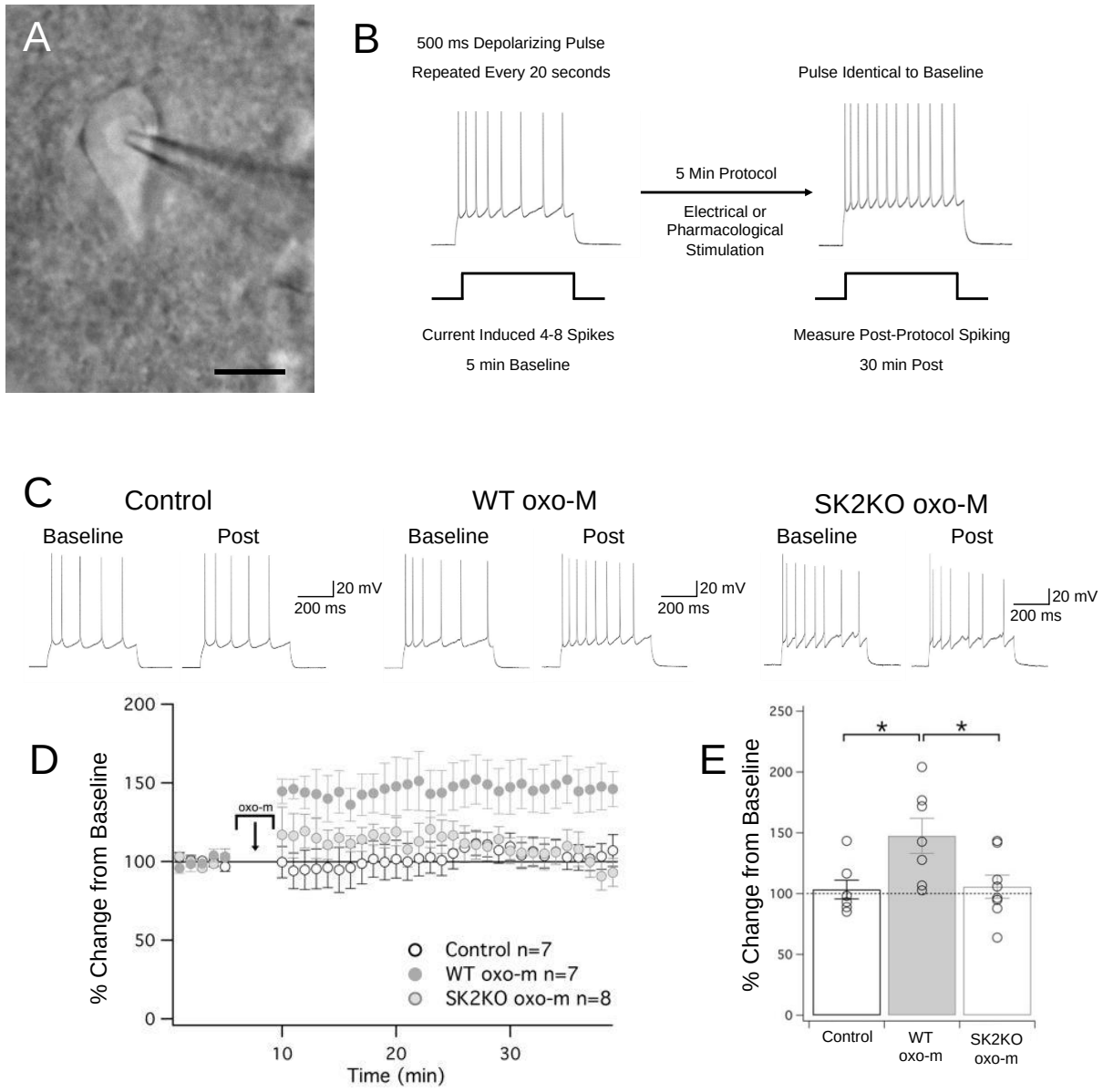

Figure 4: Muscarinic Signaling Occurs through SK2 Channels. A. Differential interference contrast (DIC) image of patch-clamp recording of a layer II/III pyramidal neuron in a slice prepared from S1 cortex, scale bar = 20 μm. B. Test protocol for intrinsic plasticity. A stable period of 5 minutes of baseline was collected where 500 ms pulses were delivered at 0.05 Hz, eliciting 4-8 spikes. Following this is a 5 min induction protocol, where oxo-m (or electrical stimulation in later figures) is applied to the slice. Following induction, cells are monitored with the same test pulses used in baseline. C. Example traces of baseline and post-induction for ACSF wash-on (control), oxo-m wash-on, and oxo-m wash-on in SK2KO cells. D. Time graph for changes in spiking relative to baseline for all three groups. oxo-m/ACSF wash on occurs from minute 5-10. E. Bar graph of each groups change in spiking relative to baseline. oxo-m significantly increased from baseline ($p=0.017$), while ACSF and oxo-m in SK2KO mice did not

(Figure 4 cont.) (p=0.68 and 0.57, respectively). Additionally, the increase observed in oxo-m was significantly greater than the changes observed in the ACSF and SK2KO oxo-m groups (p=0.021 and 0.042, respectively).

As shown in Figure 4, bath application of oxo-m caused a lasting increase in the spike count (147±14% of baseline, n=7, p=0.017). With the recording chamber design, oxo-m wash out is complete after roughly 25 min (Rinaldo & Hansel 2013), so the changes observed at the end are persistent and due to long-lasting, not transient, effects of muscarinic activation. Due to this increase, we sought to identify the source of this change in intrinsic plasticity. As discussed in Chapter 1, all SK subunits are expressed in cortex and the particular subunit(s) responsive to muscarinic pathways were not yet identified. However, given results from cerebellar Purkinje cells where SK2 channels in particular were responsible for changes in excitability, we tested where oxo-m bath application upregulates excitability in mice deficient of SK2 channels. In fact, oxo-m did not enhance the spiking of LII/III pyramidal neurons in these SK2KO mice (106±9% of baseline, n=8, p=0.57). This leads us to the conclusion that muscarinic changes in intrinsic plasticity are mediated by SK2 channels in particular, and elimination of these channels will prevent mAChR upregulation of excitability.

Electrophysiological Induction of IP Relies on SK2

Outside of pharmacological treatments, intrinsic plasticity can be triggered by repeated injection of depolarizing current pulses in cortical pyramidal neurons of intact rodents (Paz et al. 2009; Mahon & Charpier 2012). We first sought to test whether or not LII/III neurons could alter their activity in an activity-dependent manner using a stimulus protocol from Mahon & Charpier 2012. The protocol is demonstrated in Figure 5, but it involves delivering 10 depolarizing current pulses (50 ms each) at 10 Hz, followed by 2 seconds of holding current. The individual pulses were adjusted in order to evoke 1-3 spikes per 50 ms stimulus, and the entire 3 second epoch is

repeated 100 times for a total of 5 minutes of stimulation. Mahon & Charpier observed that in layer V neurons this stimulation could bi-directionally modulate the excitability of neurons, and there were cellular factors that could explain why some cells tended to go up or down in excitability (such as baseline holding current). However, the magnitude of depression was quite small (roughly 80%) and the magnitude of increase was large (150-200%), so it is very possible overall their protocol yields an increase. Later in this chapter I will discuss how their findings were controlled for in our experiments and where/if they agree/disagree.

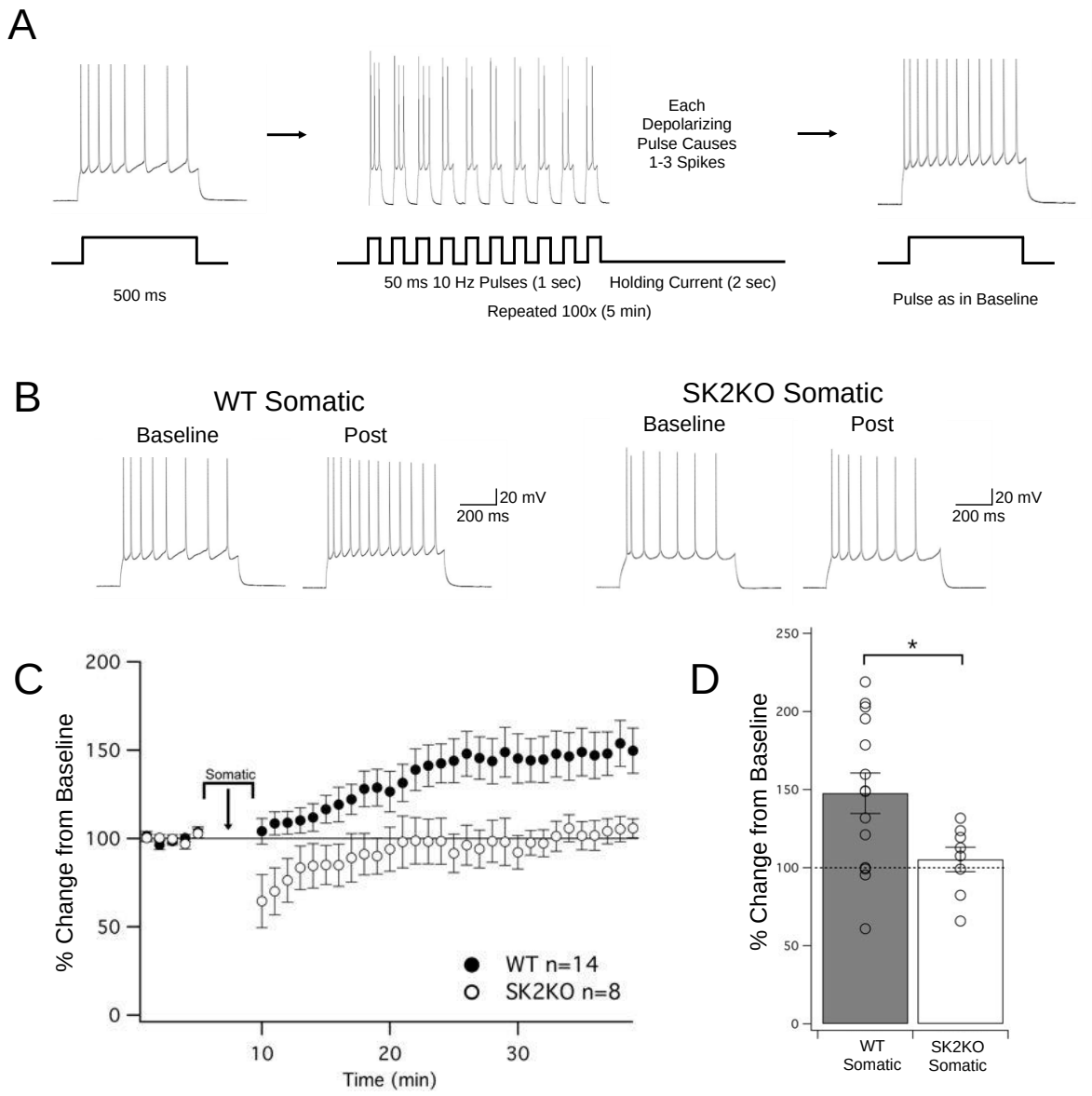

Figure 5: Induction of intrinsic plasticity through somatic depolarization is SK2 dependent. A. Protocol for inducing changes in intrinsic plasticity, modeled off of Mahon & Charpier, 2012. As before, a test pulse of 500 ms is repeated at 0.05 Hz in order to induce 4-8 spikes. Following a 5 min baseline, the induction protocol injects depolarizing current at 10 Hz (50 ms pulses), evoking 1-3 spikes per pulse for 1 second, followed by 2 seconds of holding current. This 3 second sequence is repeated 100 times for a total of 5 minutes, and then test pulses identical to baseline are again delivered to monitor for changes in excitability. B. Example traces of WT neurons and SK2KO neurons before and after the induction protocol. C. Time graph for changes in spiking relative to baseline, induction protocol occurs from minute 5-10. D. Bar graph for change in spiking relative to baseline. WT significantly increased from baseline, while SK2KO did not ($p=0.0028$ & $p=0.55$). The increase observed in WT cells was significantly greater than the changes observed in the SK2KO group ($p=0.044$).

Regardless, application of this protocol in WT Layer II/III pyramidal neurons resulted in an increase in spike count, as shown in Figure 5 (148±13% of baseline, n=14, p=0.003). Similar to Figure 5, we sought to see if changes in excitability directly involved SK channels, and SK2 channels in particular. This led us to test the protocol in our SK2KO mouse line, and here as well we were unable to find any changes caused by the protocol (105±8% of baseline, n=8, p=0.55). From these findings, we were able to show that SK2 was a key mediator of excitability, and changes in functional expression of SK2 were necessary for the effects of somatic stimulation and muscarinic activation. The next steps we took were to better understand the mechanisms by which these changes occur.

IP Induction is CaMKII Independent

We next tested for an involvement of calcium-calmodulin-dependent kinase II (CaMKII) in SK2 dependent channel plasticity, because CaMKII is one of the most abundant activity/calcium-sensors in neurons. To begin answering this question, we used a different transgenic mousseline, the T305D CaMKII mutation initially generated in Elgersma et al. 2002. More detail on this mutation is provided in chapter 1, but briefly Thr305 is substituted by a negatively charge aspartate. This mimics phosphorylation of an inhibitory site on CaMKII, whereby CaMKII is stuck in an inhibited state and cannot be activated (Rich & Shulman 1998). Since CaMKII is located in spines and associated with the PSD, we deemed it important to recruit all the synaptic machinery during our test of intrinsic plasticity (Kennedy 2000). For this reason, we adapted our somatic depolarization protocol in order to activate synaptic machinery, replacing pulses of depolarizing current with extracellular stimulation. The stimulus protocol is largely the same (10 Hz pulses, 1 sec on and 2 sec off, repeated 100 times), but an extracellular electrode was placed near the apical dendrite backfilled with ACSF and was used as the

stimulating electrode in this protocol. Current intensity was adjusted upwards until visible EPSPs yielded spikes greater than 50% of the time.

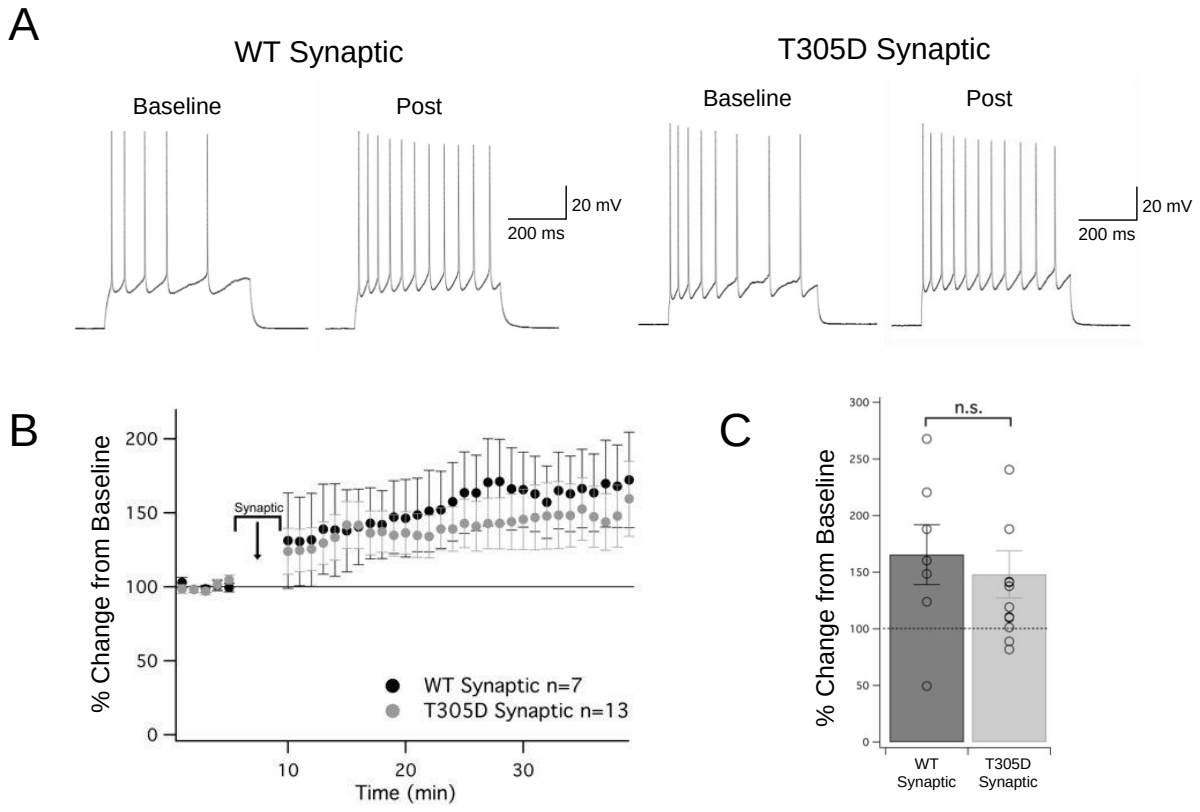

Figure 6: Induction of intrinsic plasticity is CaMKII independent. A. Example traces of WT neurons and T305D neurons before and after the induction protocol. The induction protocol was modified from somatic depolarization where an extracellular stimulus was used in lieu of somatic stimulation (further details in text). B. Time graph for changes in spiking relative to baseline, induction protocol occurs from minute 5-10. C. Bar graph for change in spiking relative to baseline. WT and T305D both significantly increased from baseline (p=0.048 and 0.040, respectively. The comparative increase between these two groups was not significantly different (p=0.27).

As shown in figure 6, our synaptic stimulation protocol efficiently triggered an increase in intrinsic excitability in WT mice (165±27% of baseline, n=7, p=0.048). The magnitude of this was largely similar to the somatic depolarization protocol, indicating that our synaptic protocol is effectively mimicking the somatic protocol and also engaging synapses (p=0.53). When this synaptic protocol was tested in T305D mice, it was also capable of driving changes in intrinsic plasticity (148±21% of baseline, n=13, p=0.040). From this we concluded that CaMKII activation was not necessary for induction of SK2 dependent changes in intrinsic excitability. WT and T305D groups were not significantly different from one another (p=0.27), although there is a potential trend and given a large enough N it could reveal a slight difference. This potential situation could possibly be explained by the fact that 10 Hz stimulation has been shown to very slightly depress cells with the T305D mutation in hippocampus. The cells were unable to express LTP, but for an unknown reason a neutral 10 Hz stimulation (neutral in WT neurons, that is) was able to induce a long-term depression in T305D neurons, reducing their activity to roughly 90% of baseline. The particular reasons and mechanisms for this effect are not known, but since a stimulus frequency of close to 10 Hz was used, it is possible we are activating a tertiary pathway that is not fully understood. However, as it stands now, the induction of intrinsic plasticity appears 100% intact in T305D neurons.

PKA and CK2 Regulate Intrinsic Plasticity

Given that SK2 channels are now identified as a mediator of intrinsic plasticity, we sought to determine if changes in plasticity are arising from an internalization of SK2 channels or a shift in their calcium sensitivity. Activation of PKA has been shown to drive internalization of SK2 channels, while activation of Casein Kinase 2 (CK2) can shift the sensitivity of SK channels to calcium/calmodulin and functionally reduce their activity. To probe the role of these

kinases, we utilized small-molecule pharmacological inhibitors to shut down activity of PKA and CK2 using H89 and TBB respectively throughout the duration of the experiment. While the drugs were in the bath, somatic and oxo-m induction protocols were performed normally, with a wash-on or stimulation occurring after collecting 5 min of baseline and then collecting 30 min of post.

Using research-grade small-molecule inhibitors opens results up to influence from off-target effects. Although selectivity for the target is known, unintended and unknown side effects can potentially skew results of an experiment. This is often lessened by screening the molecule against a variety of proteins to approximate its selectivity, but many proteins go untested so results may still be impacted by off-target effects. Another issue is that in order to better compare across literature, it is often prudent to choose a concentration of the inhibitor that has been utilized by other labs in their experiments. However, in an effort to fully inhibit the target, sometimes a concentration of drug is used that is much higher than strictly necessary given binding data. This has the potential to cause two main problems. First, it can hit more off-target elements and add additional confounding variables. There is also a risk in this situation of losing information about concentration dependent effects. For example, a large portion of the nicotine field used high concentrations of nicotine in their experiments when conducting work in slice. This became an issue after it was discovered that lower doses of nicotine and higher doses sometimes hit differential receptors and neural pathways which have different overall effects (Wolfman et al. 2018). It is entirely possible that all isoforms of a target average out when testing IC50, but that two subpopulations of the target exist whereby using the dosage recommended by IC50 and using a dose 10x IC50 yield divergent results purely based on the

effect on the target (and not side effects). These caveats are useful to consider when evaluating pharmacological data using experimental research-grade inhibitors.

Our PKA inhibitor, H89, was used at 10 μM which is the standard from literature for slice application, and is the most advanced/selective PKA inhibitor available for use. However, this is nearly 100x the IC50 of 135 nM. Additionally, at concentrations close to the IC50 of PKA, there are three known off-target hits. The first is MSK1, which is activated as part of the MAPK pathway and is involved in CREB driven translation. This potentially has an effect if any of our results require CREB translation to manifest their effects (and all muscarinic receptors can upregulate MAP kinases—see chapter 1). However, we do not currently expect any SK2 modulations to utilize CREB-dependent translation, as our current theories do not require any novel translation for effects to be seen. Another target is S6K1, which is downstream of the PLC pathway which eventually activates PKA. The exact mechanism by which PKA becomes activated is not fully known, but the current theory is that it requires PKC and calcium to work synergistically to activate it. In addition to inhibiting PKA, we must note that we are inhibiting a more upstream kinase in our pathway as well. Finally, the other known interaction is ROCKII, which is involved in cytoskeletal regulation. Hopefully this would not have a drastic impact on cellular organization given the short time exposure of the, but it is worth considering if the cellular organization of the neuron is shifted. Finally, there could be unknown binding partners to H89 that haven't been tested that have an effect. TBB has an IC50 of roughly 1 μM in rats and we used 2 μM, so the dose used is in an appropriate range. The manufacturer claims any additional interactions in their "33 Kinase Screen" bound at an order of magnitude higher than the IC50, so our TBB concentration should be fairly selective (excluding unknown interactions). The manufacturer also claims this is selective for CK2 over other CKs, but the exact magnitude

of this effect was not clear from the manufacturer. As outlined in chapter 1, it should be noted that there are potential issues with shutting down the balance of CK2 and PP2A, and functionally hitting CK2 which is bound to SK2 could be physically difficult and incur additional unwanted interactions and effects.

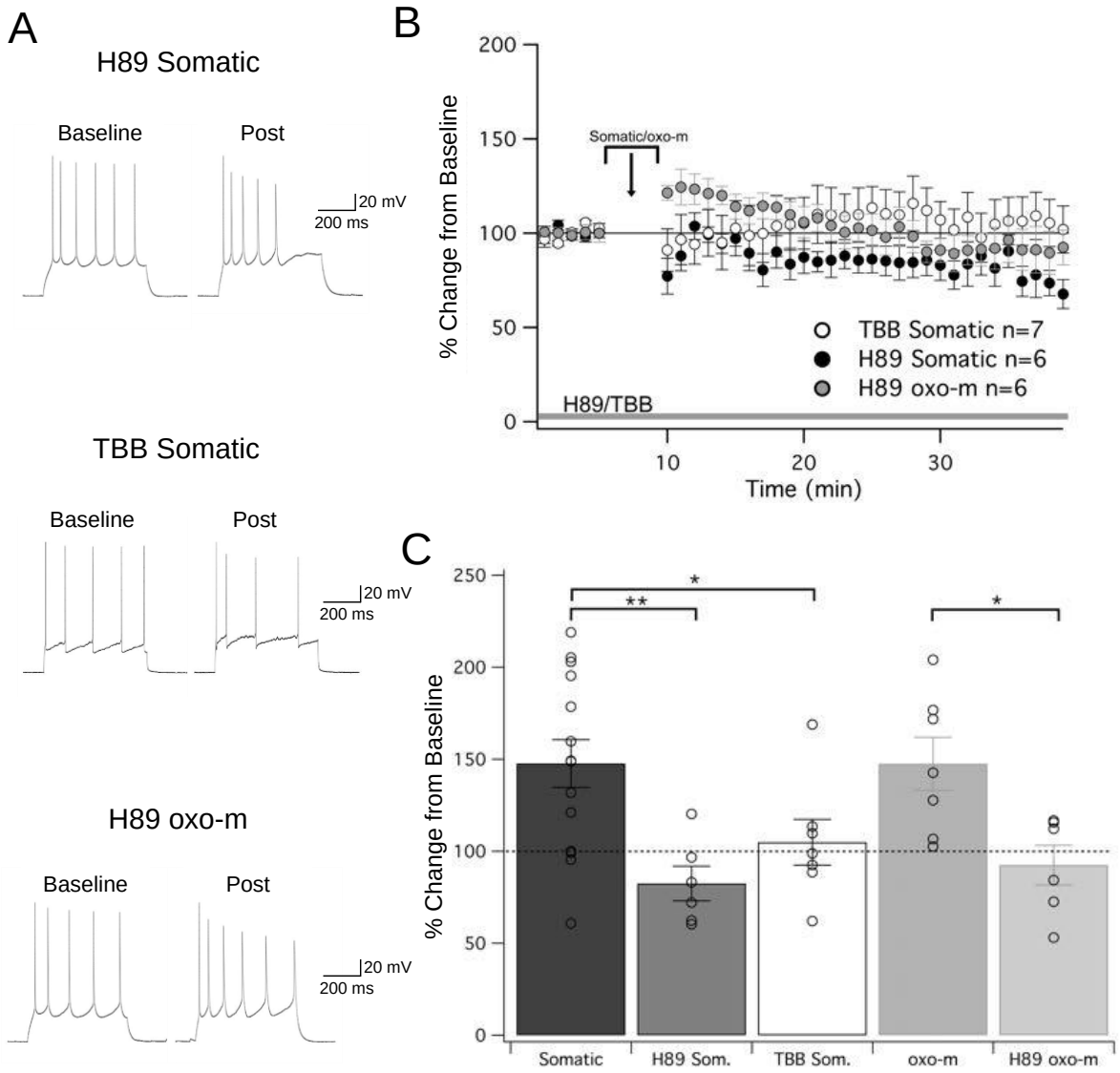

Figure 7: The role of PKA and CK2 in regulating SK2 channels in intrinsic plasticity. A. Example traces for cells treated with H89, a PKA inhibitor, or TBB, a CK2 inhibitor, that was present in the bath for the duration of the experiment. B. Time graph for changes in spiking relative to baseline. Either somatic depolarization or oxo-m was applied from minute 5-10.

(Figure 7 cont.) C. Bar graph for change in spiking relative to baseline. Neither the H89 Somatic, TBB Somatic, and H89 oxo-m were significantly different from baseline (p=0.12, 0.71, and 0.51, respectively). For the somatic induction protocol, the group of H89 and TBB cells were significantly different from control cells (p=0.007 and 0.048, respectively). For the oxo-m induction, the H89 was significantly different from oxo-m alone (p=0.039).

As shown in figure 7, bath application of the PKA inhibitor H89 prevented an upregulation of membrane excitability upon repeated injection of our somatic depolarization protocol (82±9% of baseline, n=6, p=0.12). This was not significant, but was potentially trending towards a *decrease* in intrinsic excitability. Outside of the off-target effects mentioned before, it is possible that this could be caused by insertion of more SK2 channels. There is channel turnover in the membrane consistently occurring, and shutting down the pathway involved in removal while leaving the pathways involved in insertion could result in a decrease in excitability over the recording period. Nevertheless, our results sufficiently indicate that SK2 channels are being internalized under normal conditions. In our alternate pathway, CK2 can shift the sensitivity of SK channels binding to calmodulin and functionally inhibit them. Under conditions where TBB was present in the bath and inhibited CK2, our somatic depolarization protocol was unable to cause an increase in excitability (105±12% of baseline, n=7, p=0.71). These findings taken together show that SK2-mediated intrinsic plasticity depends on the activation of PKA and CK2, and thus involves both modification and trafficking of the channels.

One potential interpretation of these results is that they are entirely consistent. PKA may play a role in activating CK2 which then can further allow PKA to phosphorylate the SK2 channel and drive internalization. This could be thought of as a positive feedback loop of inhibition, which would be one explanation of these results. Therefore, when PKA is blocked, CK2 cannot be fully activated and thus the channels remain in the membrane functioning as they are. And when CK2 is blocked, PKA attempts to activate it but fails, and potentially CK2 cannot

assist in priming SK2 to be marked for internalization by PKA. What is clear is that both kinases play a role and that the inactivation of SK2 channels is complex. This is potentially to be expected given how crowded the microenvironment of an SK2 channel is, and all of the pathways involved in regulation are just beginning to be understood. In that context, the results outline in Figure 7 are helpful in deepening our knowledge about SK2 channel regulation and offering additional data on which factors are important.

Because PKA inhibition had a slightly stronger effect, we wanted to determine whether or not muscarinic activation relied on these same pathways. While H89 was in the bath, transient application of oxo-m was unable to induce a shift in excitability (92±11% of baseline, n=6, p=0.51). This illustrates that the convergence of activity-dependent and muscarinic-dependent changes in intrinsic plasticity occurred upstream of SK2 channels and on PKA activation. This result was not particularly surprising given the role that PKA plays on SK2 channels, but it was interesting to validate that the convergence of these pathways will occur before the channels are modulated directly.

Somatic and Muscarinic Induction: Evidence for a Common Pathway

Lastly, we wanted to determine what the magnitude of change in intrinsic plasticity was when somatic depolarization was co-applied with oxo-m. When both of these protocols were applied simultaneously, it caused a significant increase in spike count (212±20% of baseline, n=9, $p=4.9x10^{-4}$) that was larger than either manipulation alone. We wondered if there were potentially any synergistic effects of activating both of these pathways simultaneously, so we mathematically combined the distributions for oxo-m and somatic depolarization. This was accomplished by adding the means of both of the increases, and then summing the variances (which can be added to combine distributions, assuming normality which both groups do not

violate). Mathematically this increase was 195±13%, and comparison to the observed 212±20% yielded p=0.49. I should note that we initially wanted to give the 'best' chance to observe any differences, so we took the aggressive assumption that we were combining all cells when using n as a value for the 'mathematical' group, which would be far more likely to reveal a false positive than a safer assumption that the smallest of the two distribution's n's would become our de-facto new n. Even under these aggressive conditions we saw no synergistic effects so we concluded these pathways merely summed. This could have indicated that both pathways were independent, but given the reliance for both on SK2 channels, this seemed unlikely. More likely is that somatic depolarization is able to activate PKA to a certain level (for example X% of PKA is activated), and this drives changes in excitability. On the other hand, oxo-m is able to fully activate every muscarinic receptor, but eventually this effect gets translated through various signaling pathways such that muscarinic activation is only able to activate somewhere around X% of PKA as well. With both of these manipulations at the same time, they are able to fully engage muscarinic signaling to turn on PKA, but also have activity dependent mechanisms allowing additional activation of PKA, so combined this can reach a new Y% of PKA activation. Under our experimental conditions, Y is approximately double X, but this could be potentially shifted around given different stimulation protocols or possibly oxo-m concentrations. In conclusion, this result could have offered insight on how two different pathways synergize, but given the flexible nature of our manipulations it definitively demonstrated that our protocols have not reached a ceiling effect.

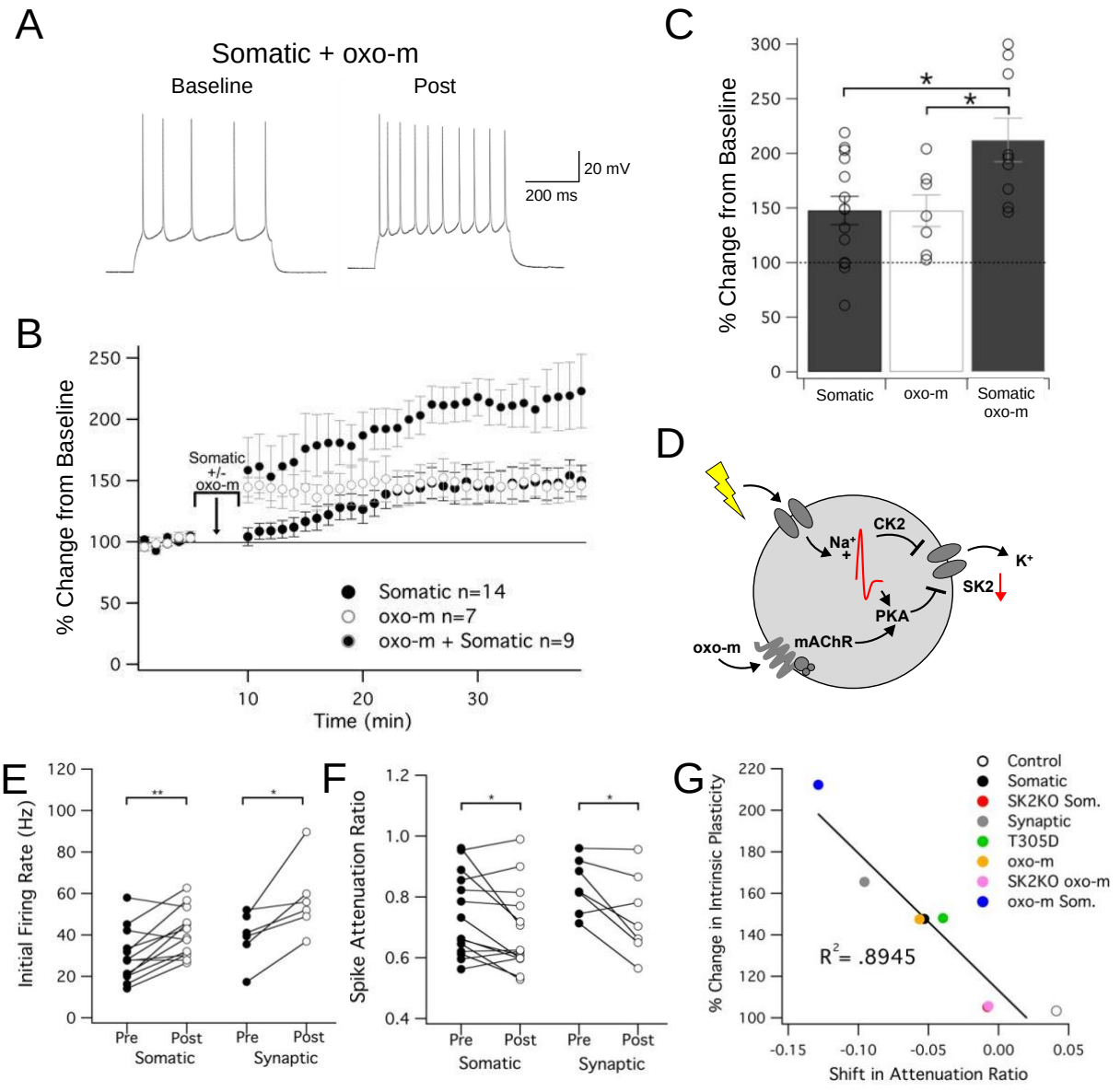

Figure 8: Intersection of somatic and muscarinic activation. A. Example trace of a cell that received somatic depolarization while oxo-m was in the bath. B. Time graph for changes in spiking relative to baseline, somatic and oxo-m stimulation occurs at minute 5-10. C. Bar graph for change in spiking relative to baseline. Combined stimulation was significantly different from baseline ($p=4.9x10^{-4}$), and significantly different from somatic or oxo-m stimulation alone ($p=0.035$ and 0.044, respectively). D. Diagram for how somatic depolarization and muscarinic pathways overlap and interact with SK2 channels. E. Difference in initial firing rate for somatic and synaptic induction protocols. Both groups increased their firing rate per sweep from baseline to post ($p=0.002$ and 0.020, respectively). F. Spike attenuation ratios for somatic and synaptic induction protocols. The spike attenuation ratio is a ratio of the spiking that takes place in the first half of the sweep, and decreases for both somatic and synaptic cell groups ($p=0.045$ and 0.034, respectively). G. Shift in attenuation ratio is strongly correlated to change in intrinsic

(Figure 8 cont.) excitability. All cells from groups which had ACSF in the bath during baseline and post are plotted, indicating a strong connection between firing later in sweeps and intrinsic plasticity ($p=3.8x10^{-4}$).

As noted in Figure 8, we then sought to characterize more completely this SK2 dependent intrinsic plasticity pathway. This led us to looking at a couple of cellular features within each particular sweep in order to better understand what changes were occurring. Our first measurement we conducted was the initial instantaneous firing rate of the first 4 spikes within responses (so chosen because baseline was calibrated to generate 4-8 spikes). The firing rate was calculated by taking the time between spikes 1 and 4 of an individual sweep, and then averaging over the baseline and post periods of each recording. The somatic depolarization group saw a significant increase in the average initial firing rate following the induction protocol (29.7±3.5 Hz increasing to 40.8±3.2 Hz, n=13, p=0.002). Similarly, the synaptic depolarization group saw a significant increase in the average initial firing rate following stimulation (39±5 Hz increasing to 57.2±7.2 Hz, n=6, p=0.02). This is not particularly surprising, given that we know in these groups the overall spike number increased. It stands to reason that since there is more spiking occurring in the same time frame, the spikes have to occur faster. While this was certainly good confirmation, we next wanted to examine where specifically spikes became more frequent.

SK2 channels can open very rapidly, but often have activation curves with a time constant of 200 ms, making them functionally quite slow as outlined in chapter 1. This led us to theorize that under normal circumstances when these channels are present, spiking starts at the beginning of the 500 ms depolarizing test pulses, but towards the end of the test pulses, SK2 channels open up and shunt off firing. That would indicate that during a shift in excitability, we would expect this late-phase ‘shunting’ and hyperpolarization to disappear and relatively more firing to take place towards the end of the sweep. To test this, we developed a metric dubbed the

spike attenuation ratio. This ratio was measured by calculating the number of spikes within the first 250 ms of depolarization, and dividing it by the total number of spikes in each sweep. Therefore, a ratio of 1 would indicate all the spiking takes place in the early phase (first 250 ms) of a pulse, and could indicate that SK channels were opened and shunted firing significantly. On the other hand, a ratio of 0.5 would indicate that spiking was uniform throughout the entire 500 ms of depolarization, and this ratio might indicate that SK channel activity was fairly weak in being able to shunt late spiking. As before, all sweeps would be tabulated and averaged for the baseline and post periods within each cell.

The somatic depolarization group saw a significant downward shift in spike attenuation (-0.053±0.024, n=14, p=0.045), indicating an increase in late phase firing in agreement with a decrease in SK2 activity. The synaptic stimulation group likewise saw a significant downward shift in attenuation (-0.096±0.035, n=7, p=0.034). Both of these indicate that late phase firing increased in our manipulations, but we wanted to be able to more directly tie late phase firing/SK2 regulation with our increases in excitability. To accomplish this, we plotted the shift in attenuation ratio against increase in excitability for all cell groups that didn't have a drug in the bath for the duration of the experiment. This includes ACSF control, somatic depolarization and oxo-m wash on in WT mice, both of the previous in SK2KO mice as well, synaptic depolarization in WT and T305D mice, and somatic combined with oxo-m wash on. When these cells were plotted, we found a strong correlation between these two factors (R^2=.8945, p=3.8x10^{-4}). Given the connection between SK channels and late phase firing, this result offers additional support that our observed changes in excitability are all strongly reliant on changes in SK2 channel function and/or expression levels.

IP Controls: No Impact of Age of Mouse, Cellular Factors, or Sex of Mouse

In looking at all of these cellular features, we sought to be aware of other factors that might impact our results. For example, Mahon & Charpier in 2012 argued that the somatic depolarization protocol could bidirectionally modulate cell responses. As outlined previously, there is a differential in magnitude that could potentially explain that overall the result is increasing excitability, but nonetheless we wanted to use their results to inform our data. Ultimately, they found that when comparing the group which increased to the group that decreased, the value of holding current was significantly different. In order to rule out any potential biasing features from our results, we monitored several cellular features shown in figure 9. Among these were the baseline holding current, average spikes during baseline, current injected during baseline test periods, and we compared spike-current response curves for WT and SK2KO groups for somatic depolarization. This last feature was somewhat difficult to compare, because during testing and finding a baseline injection current, I utilized 100 pA steps to initially find a value that elicited 4-8 spikes. Looking at figure 10, you can see that no groups are significantly different from one another, and although there is some variance across all the groups, none of the across group key comparisons had meaningfully (or statistically) different values. Therefore, while we cannot comment on whether or not in increase/decrease groups there are different cellular features, we can confidently state that we didn't bias any of our groups to increase or decrease. Directly doing that type of comparison is difficult, as we had several groups of cells with different conditions that we wanted to compare, and n's can become small when subdivided and subdivided within these groups. Mahon & Charpier took the approach of getting a very large amount of one single condition, so subdividing this group is potentially more valuable.

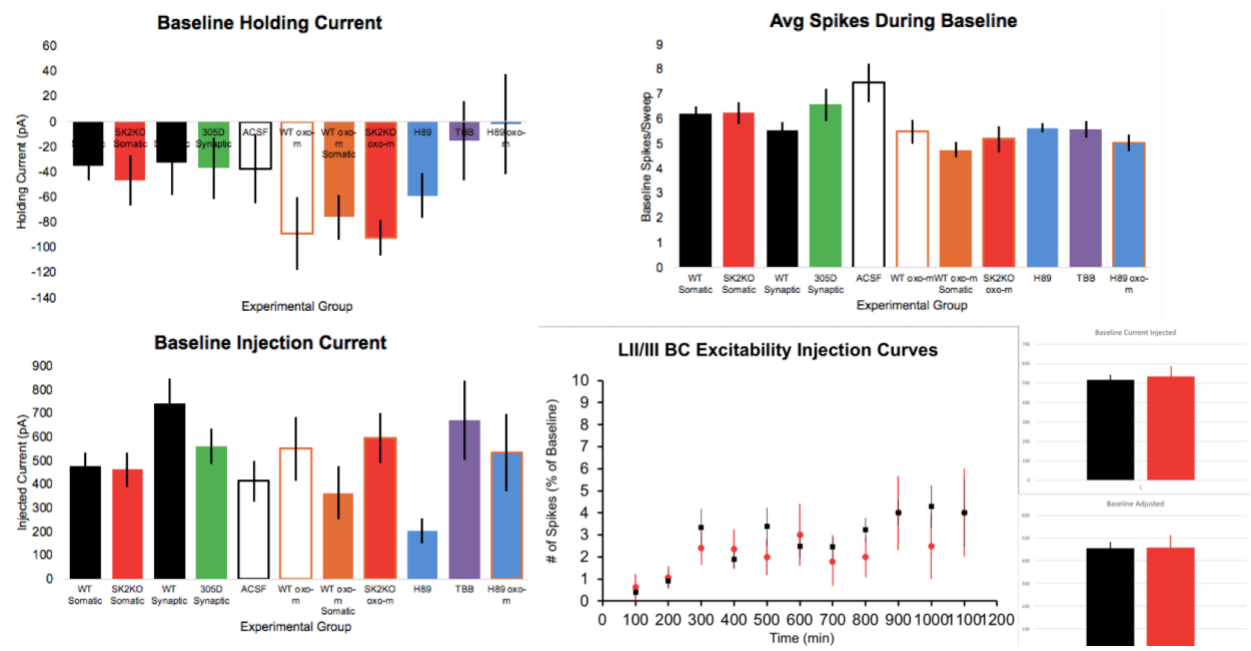

Figure 9: Cellular Feature Controls across Groups. These were cellular features we monitored in each group to ensure no one group was biased. Top left is holding current, top right is spikes during baseline, bottom left is current used in baseline (and post, for that matter), and bottom right is a current-response graph for WT vs SK2KO in somatic conditions. While there is some variation, an important thing to consider is that all important comparisons look quite similar. For example, while nothing is statistically different, WT somatic might look different from WT oxo-m. However, WT somatic is nearly identical to SK2KO somatic, and oxo-m WT is identical to SK2KO oxo-m.

To attempt to address the difficulties of using a multitude of smaller groups versus a giant single group, we had to develop a method of evaluating factors to determine if they had an impact given our numerical circumstances. Dividing small groups is difficult and can be statistically meaningless, so our solution was to group our small groups into larger, meaningful separate groups and determine if there are any differences. These groups were 1: combining all the cells in every group together, and then as a second analysis 2: combining groups which had an increase in intrinsic excitability. Therefore, we could compare these two groups, and any differences across a factor could potentially indicate that this factor was ultimately driving changes in excitability, not our specific manipulation. The two other factors we ran this analysis on were the age and sex of the mouse studied. For age, membranes are still being altered at p25-

40, although many groups still consider this age ‘adult’. What is considered an ‘adult’ mouse is often dependent on the opinion of a sub-field, but nevertheless we wanted to make sure our selected age group wasn’t skewing the data.

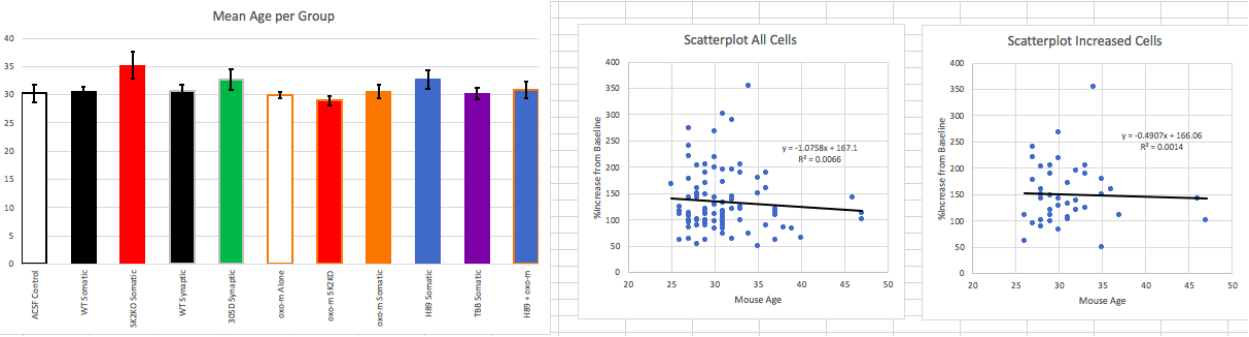

Figure 10: Mouse Age Doesn’t Predict Increases in Excitability. On the left is each groups’ average age ± SEM. On the right are correlations for mouse age against increase in excitability for all cells, and cells in the increased group.

For mouse age, we saw no differences in each of the cell groups as shown in figure 10. To further confirm that mouse age doesn’t significantly drive changes in excitability, we first plotted all cells against an increase, to test the hypothesis that age (and not our manipulations) were driving changes in excitability. There was no correlation at all here, so we also looked at just cell groups where increases were observed. This was to test the hypothesis that cell age was playing a role in the magnitude of the increase, and ultimately, we also found no correlation here. For an additional control, we ran each cell group individually (which admittedly suffered from the ‘small n’ subdivision phenomenon), and we found no significant relationships within any particular group. This led us to conclude that mouse age was not an important factor in intrinsic plasticity, at least across our particular age group. It is entirely possible that plasticity is hindered in much older mice or would be very different from our results, but within our age range there is no impact of mouse age on our results.

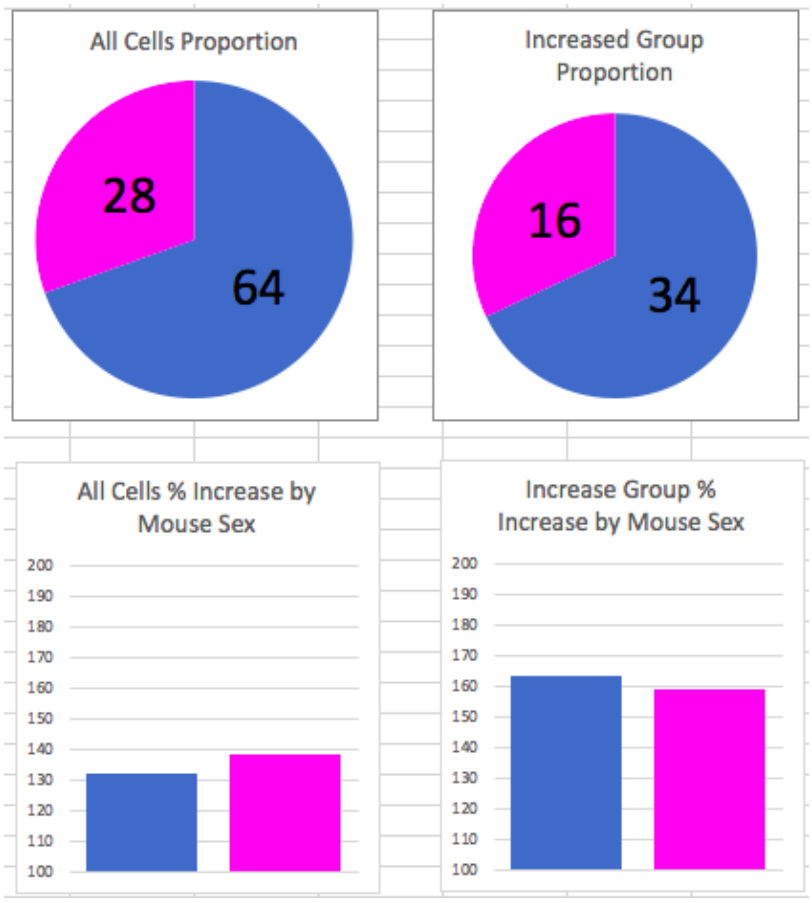

Figure 11: Mouse Sex is Balanced Across Cellular Groups. We examined the proportion of Males and Females in both the 'all cells' and 'increased cells' group and found the proportion nearly identical. Additionally, we averaged the increase across Males and Females within each group and saw no difference.

Finally, we repeated this analysis on the lines of mouse sex. Both the 'all cells' group and the 'increased cells' group were identical in the proportion of male to female mice. This rejects the hypothesis that sex of the mice biases the data in some unbalanced way. To further gain comfort around this factor not having an impact, we calculated the average increase in males and females within each group, and the percent increase across sex in each group was nearly identical as well. This allows further confidence that neither sex nor age influenced our data and skewed our results, indicating that the deciding factor in whether or not these groups could increase were our specific manipulations.

Chapter 3: Discussion, Future Directions, and Implications of Intrinsic Plasticity Experimental Results

Mechanistic Follow-Up

When it comes to future directions of a project, it can be difficult to choose which paths to follow. Solid scientific work should offer answers to previously held questions, but also spark curiosity and compel us to ask additional questions. When looking at this project, the answers which have been provided are how intrinsic plasticity can be affected by activity and muscarinic activation, and what ultimately underlies these changes (SK2 channels). Looking at future directions, there are two main categories to further examine the mechanisms and role of intrinsic plasticity. The first category is continuing to dig in on what is happening inside cells as they undergo changes in intrinsic excitability, which will be discussed first. The second is how intrinsic excitability functions on the multi-cellular level and how it can be used by groups of neurons to potentially encode information, which will be discussed in the following section.

When looking at cellular features to continue exploring, the particular timing mechanisms of intrinsic plasticity are quite interesting. For example, in looking at figure 5, if you examine WT somatic cells, they tend to start at baseline and then grow to stabilize at roughly 150%. However, the SK2KO cells have a similar timeline shape, whereby they start below baseline and then recover to baseline levels roughly 10 minutes in. This is possibly due to the protocol creating a short-term depression, from which the cell recovers. Anecdotally, during the somatic depolarization protocol, cells towards the end of the 5 minutes often struggled to fire at the same levels from the beginning of the protocol. So exactly when the SK2 channels are removed is potentially of interest, because it is possible the somatic WT cells would be responding at 150% as soon as the protocol ends, but a short-term depression is masking this. In fact, when you

examine the combined oxo-m and somatic induction, the same time-shape appears in figure 8. This increase over the first half of the post period is not seen in oxo-m alone cells (or for that matter it is not clear in TBB or H89 cells, although pharmacology could be creating issues here, so I will put a pin in these for now), so seems to be due to some side-effect of tetanization. It is entirely possible that this theoretical tetanization induced depression is merely an experimental artifact with no physiological relevance, but layer II/III and V neurons in cortex have shown that they sometimes undergo a decrease in excitability before an increase (Glazewski et al. 2017; Kida et al. 2016). Perhaps this decrease is occurring and is valuable in inducing changes in excitability, so understanding the mechanisms that govern this timing effect could be interesting in uncovering the cellular pathways.

Another place to continue examining is the cellular mediators of SK2 channel functional downregulation. Our results argue that PKA and CK2 are necessary for expression of intrinsic plasticity, however these are caveated by some of the discussion in chapter 2. Pharmacological tools are not perfect, so more precise methods might be able to untangle the signaling pathways targeting SK2 channels. For example, SK2 cannot be internalized if residues are blocked that prevent PKA phosphorylation. Perhaps using a peptide that blocks these or generating transgenic mice with modified SK2 receptors that prevent internalization could allow sharper testing of this PKA hypothesis. The mechanisms by which PKA becomes activated through muscarinic signaling are still unknown. PKA is often thought of as the downstream target of G_s signaling, but muscarinic receptors do not directly activate this pathway (and M_2/M_4 oppose this pathway). It is currently thought that PKC (activated through G_q via the PLC pathway) and calcium may coordinate in some way to activate PKA, but determining the actual steps of activation could be very useful in understanding how it eventually targets SK2 channels.

Finally, understanding CK2 more thoroughly would be very valuable in seeing how it may or may not interact with PKA to shut off SK2 channels. Our results indicate that both PKA and CK2 are necessary, although how these two kinases interact directly is not understood. Answering this question might be trickier, as getting access to SK2 can be difficult given how crowded the channel microenvironment is. Perhaps PP2A could be inhibited as well in order to measure how this competitive phosphorylation and de-phosphorylation process of calmodulin occurs in greater detail. Either way, there is clearly a level of interaction occurring that cannot be completely explained by classic theories of PKA being entirely internalization and CK2 shifting sensitivity with zero overlap, unless we assume pharmacology is introducing some level of error. As discussed previously, research-grade small molecule inhibitors consistently struggle with specificity, and the micro-environment of SK2 channels might make accessing molecular targets more difficult.

Intrinsic Plasticity as a Mechanism of Engram Encoding

Instead of examining what happens on the single cell level in greater depth, it is also possible to extrapolate these results to a multi-cellular level. This includes some of the concepts outlined in Titley et al. 2017, and with this knowledge some of these hypotheses can begin to be tested. Revisiting some of the work done by the Tonegawa laboratory, they were able to show that a fear memory can be sufficiently encoded by activating a group of previously coordinated neurons (the engram). Furthermore, they showed that this memory is encoded even in the absence of LTP, given that the neurons become active in the same initial pattern (Liu et al. 2014; Ryan et al. 2015). With the lack of observed synaptic plasticity, and considering the fact that the stimulus used to re-engage the engram is a fairly blunt ChR2 driven somatic stimulation, intrinsic plasticity is an intriguing potential mechanism for this encoding. Merely shifting the

sensitivity to inputs would allow a large group of neurons to more easily coordinate their activity in the future and thus activate the engram given a single, priming signal to a large group of potential neurons. Our results show the importance of SK2 channels and muscarinic signaling/activity-based activation to the induction of intrinsic plasticity, and this knowledge could be used to determine if IP helps encode engrams.

A very straightforward way to test this hypothesis is to examine engram formation in WT versus SK2KO mice. An initial test could be to look at a particular type of memory that we know has an engram encoding, such as location/context in hippocampus or visual responses in visual cortex, and track the formation and stability over time. A particularly well-suited method to this could be using GcAMP6 (either viral or transgenic) expressed in pyramidal cells of hippocampus or cortex, and using in-vivo imaging to see cellular activation. Using a virtual environment, the particular cluster of cells that encodes for a particular location could be isolated and monitored over time (perhaps a couple of days initially, because currently long-term monitoring through cranial windows is quite difficult, although future technological developments could potentially ease this). Then the experiment could be repeated in SK2KO mice, and differences in how engrams are activated within a context or their stability over time could be tested. One potential issue with this line of experiments is that the SK2KO mouse line was generated by replacing exons 1 and 2 with a non-functional eGFP. This was at least the theory, and was presumably done in order to make testing for successful KOs more straightforward, so having or not having eGFP was not relevant. However, when using another fluorescent tool, as many of our current methods require, this extra signal could prevent experimentation if the eGFP is functional. Preliminary research in the Hansel lab is examining if there is residual eGFP signal in SK2KO

mice which could allow these mice to be used in this way, but technological innovation will continue and presumably this hurdle will be passed.

Another variant on this type of examination could be done in barrel cortex, and examining the clusters of cells that respond to whisker deflections. Barrel cortex, which is located in primary somatosensory cortex of mice/rats, has particular regions with denser cell bodies that on average tend to respond best to a particular 'primary' whisker deflection/stimulation. However, inside a particular barrel, individual cells have varying response properties and only a small portion of cells actually respond to the primary whisker. Even within this group, the probability that a particular tuned cell will respond to a whisker stimulation can be 10% or lower (Wang et al. 2019). When taken as a group, all of these neurons together as a group could potentially encode the response to the whisker. As outlined in the hippocampus, our results surrounding SK2 could also be used to see if this engram formation is damaged in response to disruption of SK2 channels/muscarinic signaling. However, given the ease and repeatability of stimulation, additional experiments could be considered that could more directly exert control over engrams given intrinsic plasticity is a mediator of encoding. For example, we validated a somatic depolarization protocol that induces intrinsic plasticity. Using ChR2 alongside GcAMP6, it could be possible to identify a cell expressing ChR2 which is not part of the engram corresponding to a particular whisker (it should be noted that the lab of Rafael Yuste has conducted experiments in a similar manner, using these tools to monitor and manipulate engrams). Then, using the somatic depolarization protocol, the non-engram cell could be stimulated and intrinsic plasticity could be induced. Further monitoring the region of interest, you could determine whether this new cell was also 'induced' to join the engram, which would be expected if SK2 dependent intrinsic plasticity underlies the encoding. Also, by enhancing

muscarinic signaling (perhaps pharmacologically), the time-course or strength of engrams could be monitored. Overall, the purpose of this section is not to exhaustively list every experiment that could be conducted to study how cells group together to form engrams. However, our results now have dictated several learning rules that can and should be tested on how engrams form on a multi-cellular level.

A further step in developing this concept is not how intrinsic plasticity encodes engrams, but how engrams encoded by intrinsic plasticity could be activated. Changes in intrinsic excitability have been shown to enhance the fidelity of spiking and better synchronizing the timing of a spike given an incoming EPSP (Sourdet et al. 2003). On the multi-cell or even single-cell level, it could be explored how SK2-dependent plasticity allows for activity to become more easily synchronized and group cells together. Looking at EEG, which of course suffers from a lack of resolution, most of the activity during waking hours is averaged out to a relatively stable signal as large-scale coordination is absent. However, during sleep phases the activity becomes coordinated as waves of electrical activity roll through cortex. Looking at cells which have undergone SK2-depedent plasticity, it is possible that this coordinated signal causes synchrony among the selected group of neurons and allows further consolidation of the memory. This theory could fit well with the well-known observation that sleep allows for memory consolidation. Overall, it would be very valuable to determine more effectively what triggers intrinsic plasticity in mice (given that the somatic depolarization protocol is not typically what a cell will receive under normal conditions), but also how this shift in excitability allows for the synchronized activity among the participating neurons. Measuring if synchronized activity (such as that seen in EEG data, but potentially measured using LFP or other methods) drives cellular activity, specifically calling out an engram encoded by changes in intrinsic excitability, could

offer a useful view of engram encoding. And further, it would be interesting to identify various sources of synchrony (such as sleep or short-term attention related nicotinic signaling) and how these sources interact with groups of cells that have undergone changes in intrinsic plasticity.

Interactions Between Intrinsic and Synaptic Encoding

Additional studies could be conducted on how intrinsic plasticity coordinates with synaptic plasticity. Intrinsic and synaptic plasticity may possibly serve entirely different functions, with each type of plasticity responsible for mutually exclusive tasks. However, this is unlikely given that intrinsic plasticity can directly modulate EPSPs which can drive synaptic plasticity. When the results of Ryan et al. 2016 are also considered, LTP was required for recall of a memory while cellular excitability could activate a 'latent' engram, indicating that there is overlap between the two. Given that there is some overlap, it worthwhile to examine what exactly this overlap is and how these two mechanisms synergize to effectively encode information. For example, IP could tag a group of cells to represent an engram, and this induces synaptic connections to be formed among the group in order to fully consolidate the memory. Under these conditions, both IP and LTP work together to encode a memory, and going forward either could be shifted/modulated to adjust the memory. However, it could be that intrinsic plasticity could be induced as a long form of short-term memory, whereby it helps synaptic contacts form a synaptic network to become a linked, stable engram. Then, after a day or two, the intrinsic plasticity could fade back to baseline as the synaptic network remained. This would mean IP is required for memory formation and consolidation, but not for very-long term recall. Or another phenomenon exists in memory, such that a task when forgotten will be easier to re-learn than to learn initially, indicating that a 'trace' of the memory still exists in the brain. Perhaps this takes place by leaving weak synaptic contacts among an engram that encodes the

memory, or perhaps there is an aspect of these neurons still tagged by intrinsic plasticity that allows easier reactivation. Regardless, understanding how these two different types of encoding (LTP and IP) function and what roles they play in memory will be necessary in understanding their importance.

In conclusion, figuring out how IP works on a multi-cellular level is a vast project with large implications for how brains process memories. Untangling the pathways inside a cell is also no easy task, but will be necessary to understand specifically how IP works. Both these tasks will offer greater insight into how neurons behave, but it might also allow for improving artificial intelligence and neural-like computations. Many of the current rules that dictate 'neural-nets' or computers built more like brains fall into categories of supervised and unsupervised learning. Supervised learning is using known inputs and outputs to tweak a system, so will be unaffected by principles like IP. However, many unsupervised learning methods use principles like LTP to adjust 'synaptic weights' inside the computer. Discovering more rules such as IP may not only allow for better understanding of humans, but also better creation of machines as rules based on these new forms of plasticity become understood.

Chapter 4: Autism Spectrum Disorder, 15q11-13 Duplication, and Epilepsy

Introduction to ASD and Dup15q

Autism Spectrum Disorder (ASD) is a condition that affects 1 in 68 children and can manifest in a variety of behavioral and intellectual disabilities (Christensen et al. 2018). ASD is typically characterized by deficits in social interactions, compulsive or repetitive behaviors, and difficulty with communication. Unfortunately, in addition to cognitive deficits, a subset of patients diagnosed with ASD also develop and suffer from non-cognitive deficits. These can include hyper- or hypo-sensitivity, motor/coordination problems, or seizure disorders that can be even further debilitating. Although many links to particular genes or factors have been found that increase the likelihood of ASD, such as Fragile X or mothers who contract an infection during pregnancy, a comprehensive mechanistic understanding remains elusive (Eshragi et al. 2018). In order to study ASD on a more cellular level and have more control of experimental paradigms, animal and mouse models have been developed in order to examine ASD more thoroughly. While there are certainly issues with modeling a condition with defining features of some of the most complex aspects of human behavior (i.e. communication), more direct lines can be drawn from mimicking genetic forms of ASD.

In particular, a genetic mouse line was made to model of the most common chromosomal aberration in patients with ASD, Dup15q syndrome. Dup15q is a duplication of the 15q11-13 region and is responsible for 0.5-3% of cases of ASD (Scoles et al. 2011). This duplication, when inherited maternally in humans, is penetrant at a very high rate with up to 90% of carriers showing symptoms. However, when inherited paternally, this rate is lower but still might be as high as 50% (Cook & Scherer 2008; Urraca et al. 2013). It should be noted however that the causes and strength of this imprinting is not very well understood. For example, the best

estimates of penetrance from the 90% maternal and 50% paternal come from a study of 10 patients with a maternal duplication and 4 with a paternal duplication. Answering this question of rate can be difficult as recruiting for studies will likely overestimate rates due to the fact that symptomatic patients with a duplication are more likely to be registered than a typically developing individual who may or may not know they have a duplication in the first place. Given the high rate and severity of symptoms, this would potentially limit this risk for error, but it is still possible.

In order to better study ASD and Dup15q, a mouse model was developed that mimics the chromosomal regional duplication by the laboratory of Dr. Toru Takumi at the RIKEN Brain Science Institute. The region of mouse chromosome 7 (which is syntenic to human chromosome 15) was duplicated as outlined in figure 12 (adapted from Nakatani et al. 2009). Outside of the model being a different species, it is worth noting that the human duplication typically occurs differently from how the mouse was generated. The more common form of the duplication is an interstitial duplication in which the genetic stretch is inverted when duplicated. More rarely and severely is an isodicentric duplication, in which the region is duplicated twice and becomes an extranumerary chromosome. The mouse duplication was generated by duplicating the region and repeating it in the same order as before. In order to validate this model, the mice were tested for classic phenotypical presentations of ASD seen in humans: social interaction differences. In this case, the mice were placed in a 3-chamber testing arena with the middle chamber empty, and each side chamber holding a novel/familiar mouse or object inside of a smaller cage. When comparing a novel mouse to a familiar one, or a novel mouse to a novel object, WT mice will prefer the novel mouse and spend more time interacting with it (or around its cage, to be more precise). When these mice were tested, those with a paternal duplication (patDp/+) showed no

difference in preference, offering some initial support for this model. However, the maternally duplicated mice (matDp/+) did not show any differences from WT, and this imprinting pattern is reversed from what is seen in humans for not yet understood reasons. There are of course additional caveats, given that the very complicated thing being modeled (human social interaction) is clearly not a 1:1 correlation between mouse exploration and interaction. And the mimicking of deficits in mouse social interaction is a good beginning point, although the inversion of imprinting phenotype can create problems for the model. For this reason, it could be of interest in further validating the model (and studying the phenomenon of seizures more directly) if these mice mirrored another phenotype seen in Dup15q in humans: epilepsy.

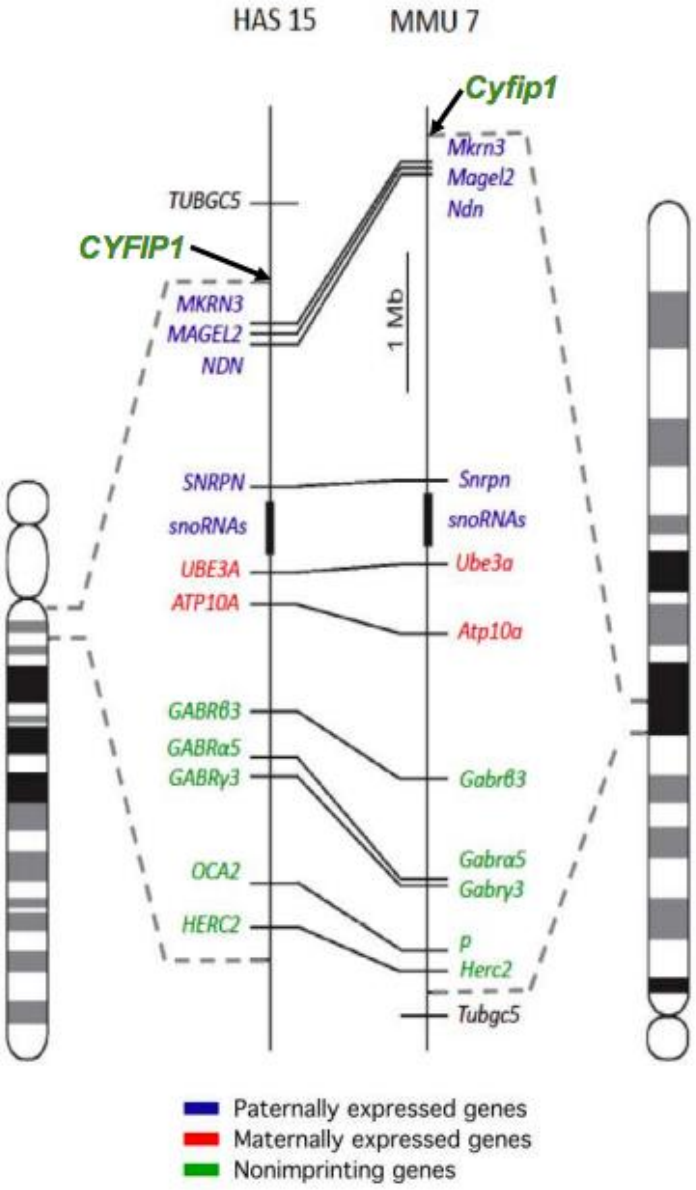

Figure 12: Human chromosome 15 and mouse chromosome 7. Adapted from Nakatani et al. 2009, this figure shows the chromosomal region duplicated in Dup15q and the syntentic region of mouse chromosome 7.

Introduction to Seizures and Epilepsy

In addition to the social and cognitive deficits seen in ASD, there is a significant overlap between ASD and epilepsy. Up to 20-30% of children with ASD also have epilepsy, and

population-based studies have found that a diagnosis of epilepsy confers a 10-22 times higher risk of an additional diagnosis of ASD (Tuchman et al. 2010; Selassie et al. 2014; Sundelin et al. 2016). This is, of course, much higher than the general population, but the overlap of Dup15q and epilepsy is even stronger. Human patients with Dup15q have a high incidence of infantile spasms, and up to 65% receive an additional diagnosis of epilepsy (Conant et al. 2014). This is particularly striking given that it is higher than many forms of syndromic autism, such as Fragile X, where still 10-20% of patients have seizures (Berry-Kravis 2002). While seizures are somewhat notorious among neuropsychiatric disorders as having current therapies which are largely efficacious and tolerable, there is still a portion of the population that cannot control their seizures and/or struggle with side-effects of existing medications. This problem is noted frequently in patients with Dup15q who have seizures, so understanding how seizures originate in this population may help the seizure-resistant population at large. Additionally, understanding how seizures develop in Dup15q may help understand how ASD and epilepsy interact more generally. This could generate insights into both conditions, potentially identifying an abnormality in neural circuitry that explains both.

Epilepsy is often explained by the theory of "excitation-inhibition imbalance" which has explained a large portion of how seizures occur and propagate. This theory of seizures was developed from pharmacology, whereby giving a patient a drug that increased inhibition could stop seizures and giving a patient/subject a drug that blocks inhibition or increases excitation causes seizures. The overall concept stated that the brain was sitting at an optimal balance point of excitation and inhibition, and that an imbalance of excitation over inhibition would cause seizures. Given the large treatment success in humans, this theory has yet to become discarded, although it has deepened considerably since its inception. For example, with EEG, it became

possible to detect large scale synchronized activity in cortical areas. This allowed researchers and physicians to observe that seizure activity was also extremely synchronized, leading to a theory that hyperexcitability was leading to loops of excitation that were not being shut off. While there are concepts that still involve local neural circuits to drive this outcome (that I will explore more as I discuss results from slice), this concept continued to be updated in Smith & Schevon 2016. They outline the most modern twist on excitation/inhibition theory, which correlates seizure activity across all brain regions to identify positive feedback loops that don't require hyper-local excitation circuits, but can instead span the brain. Finally, before delving into the cellular level and our understanding of the circuitry, I will briefly mention the brain areas involved. Seizures can range from 'classic' tonic-clonic seizures, to more localized and/or absence seizures. Overall, seizures typically propagate across cortex, so cortex becomes involved eventually in the seizure. Focal epilepsy, where only a particular brain region will initiate or propagate a seizure, is roughly 30% of epilepsy (Manford et al. 1992). Broken down further, frontal lobe epilepsy is roughly 20-30% of focal seizures and temporal lobe epilepsy (which can span several cortical regions and involve the hippocampus) accounted for 30-40% of focal seizures, with the rest somewhat uniformly spread around cortex. Given the low relative rate of focal seizures and widespread origins of focal seizures, we chose somatosensory cortex as our cortical region of interest and conducted our experiments there.

Looking particularly about what happens in the brain during a seizure, the general timeline is displayed in figure 13. Seizures have an 'ictal wavefront' from which the seizure can originate and propagate across the cortex, and using an implanted multi-electrode array Smith et al. were able to observe the anatomy of a seizure in humans. Initially as the wavefront approaches, cells begin to get recruited into the synchronized activity of the wavefront and

synchronized activity can be observed. Once the wavefront reaches the cortical area, it drives synchronization of activity in the ictal core (inside the wavefront) and continues to propagate the wavefront across cortex. Eventually, firing becomes less synchronized and loses its particular timing, where the seizure will end.

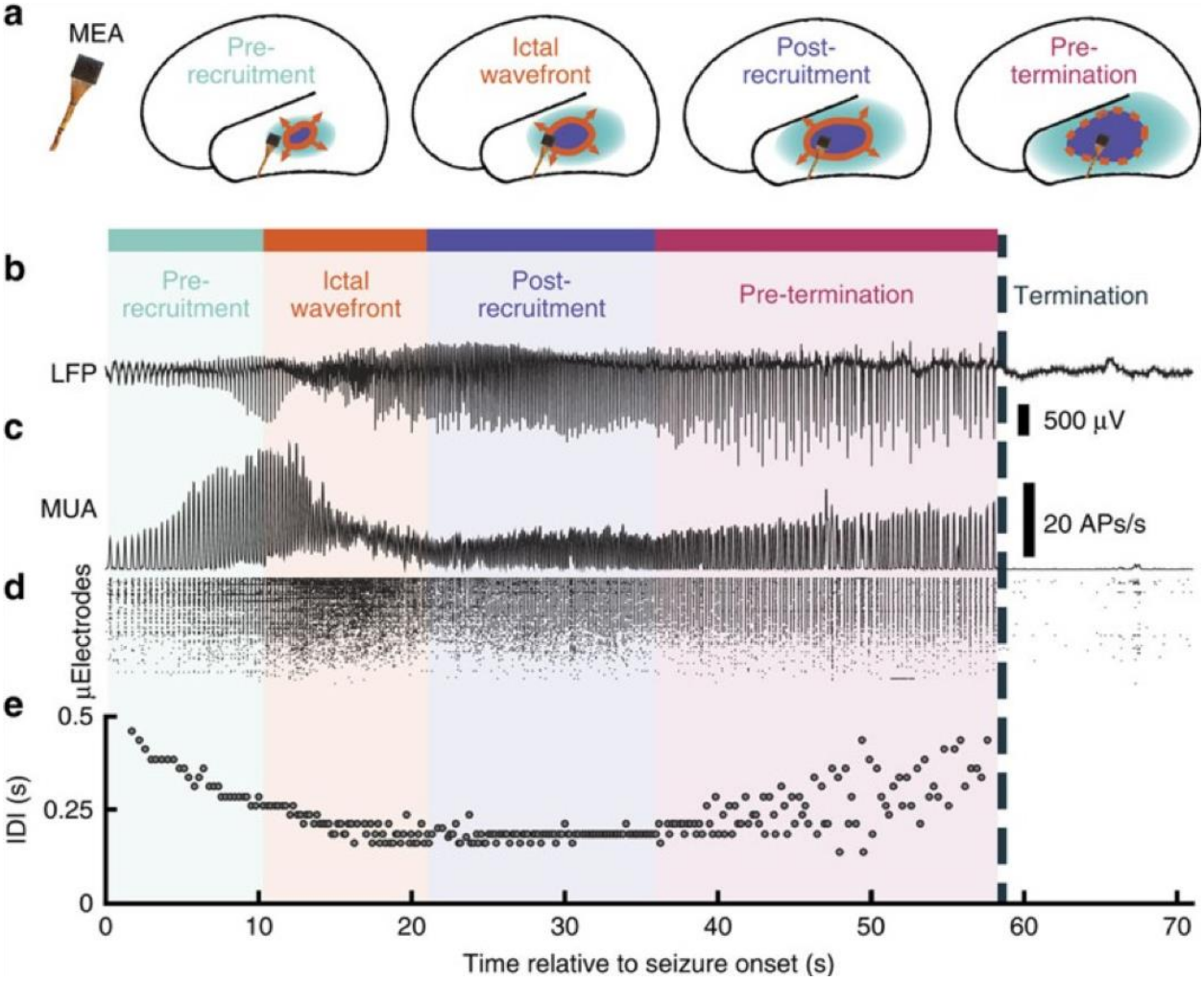

Figure 13: Dynamics of the ictal wavefront in seizures. Figure from Smith et al. 2016 in *Nature Comm.* outlining how seizures propagate across cortex.

Given that the ictal wavefront is extremely important in driving seizure activity, it is worthwhile exploring what is occurring on a cellular level in slices. Trevelyan et al. in 2007 illustrated that layer V neurons were both generating and propagating epileptic activity in slices.

After patching three layer V cortical neurons in visual cortex, they were able to simulate epileptic tissue using a 0 Mg^{2+} concentration in solution and monitor the cell's activity. When ictal events began propagating throughout the slice, they could monitor the path of these events by seeing the order in which the neurons were recruited. For example, they could see a large ictal event hit neuron A, then B, then C. Afterwards, they saw characteristic rhythmic activity in all three neurons, which was reversed as the seizure activity propagated backwards. Specifically, they could now observe a spike in C preceding a spike in B and then A. The conclusion is that this ictal activity was generated and carried by layer V neurons, which led us to explore these neurons in more detail.

In order to specifically determine if our patDp/+ cells had an increased propensity to seizure, we sought to identify a cellular correlate we could examine. Many potential correlates exist, with various pros and cons for testing in our model. For example, layer V pyramidal neurons in a rat model of epilepsy where tissue is damaged has sometimes shown an increase in spontaneous EPSCs, a decrease in spontaneous IPSCs, increase in overall firing rates, or even counterintuitively a decrease in EPSC amplitudes (Takahashi et al. 2016; Nichols et al. 2015; Jin et al. 2006; Ping & Jin 2016). These results sometimes agree with one another and human level data, such as finding layer VI neurons of epilepsy patients with deficits in GABA receptor subunits (Loup et al. 2009). However, sometimes these observations are not useful in identifying a cellular correlate, such as the finding that cortical neurons from epileptic patients do not have deficits in plasticity (Wierschke et al. 2010). This may be relevant to understanding epilepsy, but does not offer a cohesive test to determine if a particular cell is prone to generate seizures.

Another issue with some of these features is that they take a known model of seizures and identify cellular correlates with the observed phenotype. In our case, we have not yet observed

seizures in our model (which will be discussed in a later section), so any potential finding of synaptic alterations wouldn't necessarily confer a direct connection to seizures. After conversations with Wim van Drongelen, we identified paroxysmal depolarization shift (PDS) as a cellular correlate of interest. Initially defined in the context of EEG, PDS referred to an observed depolarization block in an individual cell following a single seizure-like spindle in EEG signal (Schwartzkroin & Pedley 1979). The van Drongelen Lab had also noticed this PDS in cultured epileptic tissue, and played a very important role in driving ictal activity (Tryba et al. 2019). For this reason, we sought to determine the threshold for activating PDS in layer V pyramidal neurons of patDp/+ mice compared to WT mice. This involved patching in whole-cell current clamp, injecting increasing current until the cell couldn't continuously fire (indicating depolarization block and PDS). If the threshold for this seizure-like PDS response was lower, it could potentially indicate that this tissue was more susceptible to seizures.

Materials and Methods

Thalamocortical slices (350 μm) were prepared (orientation described in Agmom & Connors, 1991) from postnatal day P25-40 mice after isoflurane anesthesia and decapitation. This procedure is in accordance with the guidelines of the Animal Care and Use Committee of the University of Chicago. The slices were cut on a vibratome (Leica VT1000S) using ceramic blades. The slices were cut in a sucrose slicing solution containing the following (in mM): 185 Sucrose, 2.5 KCl, 25 Glucose, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, and $MgCl_2$, bubbled with 95% O_2 and 5% CO_2. Following slicing, the slices were kept in artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose, bubbled with 95% O_2 and 5% CO_2. The slices were allowed to recover for at least 1 h, and were then transferred to a submerged recording chamber superfused with ACSF at near-

physiological temperature (31-34°C). Whole-cell patch-clamp recordings were performed under visual control using a 40X water-immersion objective mounted on a Zeiss Axioskop 2FS microscope. Patch pipettes (2.5-4.5 MΩ) were filled with internal saline containing the following (in mM): 9 KCl, 10 KOH, 120 K-gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 Na_2ATP, 0.4 Na_3GTP, and 17.5 sucrose, pH adjusted to 7.25. Patch-clamp recordings were performed in current-clamp mode (capacitance cancellation switched off) using an EPC-10 amplifier (HEKA Electronics). Membrane voltage and current were filtered at 3 kHz, digitized at 10 kHz, and acquired using Pulse software (HEKA Electronics). After whole-cell patching layer V pyramidal neurons of primary somatosensory cortex in voltage clamp, series resistance was measured and bridge compensated once in current clamp. Before recording in current clamp, a bias current was applied to prevent spontaneous spike activity and to set baseline voltage to -70 mV. Input resistance (R_i) was measured by injection of hyperpolarizing test currents (100 pA, 100 ms) and was calculated from the voltage transient toward the end of current injection.

PDS threshold was determined by injecting depolarizing current for 500 ms every 10 seconds (0.10 Hz) in an increasing fashion, beginning with 50 pA and increasing with 50 pA steps until PDS was reached. PDS was defined by observing the cell responding completely to injected current (spiking throughout), and then at levels above this current stopping being able to track current injections. The mice used in the experiments are patDp/+ mice received from the Takumi laboratory and bred in the University of Chicago animal facilities. Control mice used were WT littermates or C57 mice on an identical background to the WT littermates. Data analysis was conducted by running an unpaired student's *t*-test on the values for PDS threshold in patDp/+ and WT groups.

Experimental Results of PDS Measurements

When we began our experiments recording from layer V pyramidal neurons of somatosensory cortex for WT and patDp/+ mice, we first monitored if there were any difference in baseline metrics. We didn't find any difference in membrane resistance (58±4 and 57±4 MΩ for WT and patDp/+, respectively) or holding current injected in order to hold the cell at -70 mV during non-stimulation periods (-135±31 and -66±23 pA respectively). I should also note that while the values for resistance are nearly identical, holding current is almost close to significantly different (p=0.08). However, the magnitude of this result is not particularly meaningful. The threshold for PDS, which is roughly 1500 pA, is much higher than the potential difference of roughly 60 pA between the holding current for these two groups (Figure 14). If there is a statistical difference therefore, it may be interesting but it does not have a meaningful impact on the measurement of PDS. To test for PDS, 50 pA of current was injected and increased in 50 pA steps, as the cell was monitored. Before reaching PDS, we recorded the value of current injection where the cell first spiked as an approximation of rheobase. We again noted no difference in the threshold to spiking between WT and patDp/+ cells (319±31 and 312±34 pA, respectively).

However, when we recorded the threshold at which the cells cannot follow firing and undergo PDS, we observed a significant difference in threshold. As noted in figure 14, the threshold for WT cells to undergo PDS is 1820±120 pA versus only 1400±70 pA in patDp/+ cells. This result offers evidence that these cells have a reduced threshold to exhibit a seizure-like response in PDS, and could indicate that patDp/+ mice have an increased propensity to seizures. To gain more confidence in our results, we also tested the potential influence of sex and age in our groups. When looking at male vs female mice, we found no impact on PDS (1720±120 &

2020±180 pA, respectively M vs. F, p=0.22) or rheobase (293±32 and 297±26 pA for M vs. F). When correlating PDS and rheobase with mouse age, we again found no relationship (R^2=0.067 for PDS and 0.016 for rheobase). Lastly, as a measure of curiosity and an attempt to better characterize PDS, we examined whether or not there was a relationship between PDS and rheobase. One potential outcome could be a strong correlation, indicating that if a cell spikes easier it will also have PDS easier. Another outcome, which we eventually found, is that these two cellular measures are unrelated to one another (R^2=0.142). Our result showing a decreased threshold for PDS could potentially offer additional validation of PDS as a measure for epilepsy, as every other cell feature appeared relatively normal supporting normal function. However, because the threshold for activating PDS is lower in patDp/+ than in WT could indicate that the threshold for seizure generation is lower in the patDp/+ mice. This result is interesting on a cellular level, but of course PDS is still being validated as a marker of epilepsy and a large question here is whether or not these mice even have seizures.

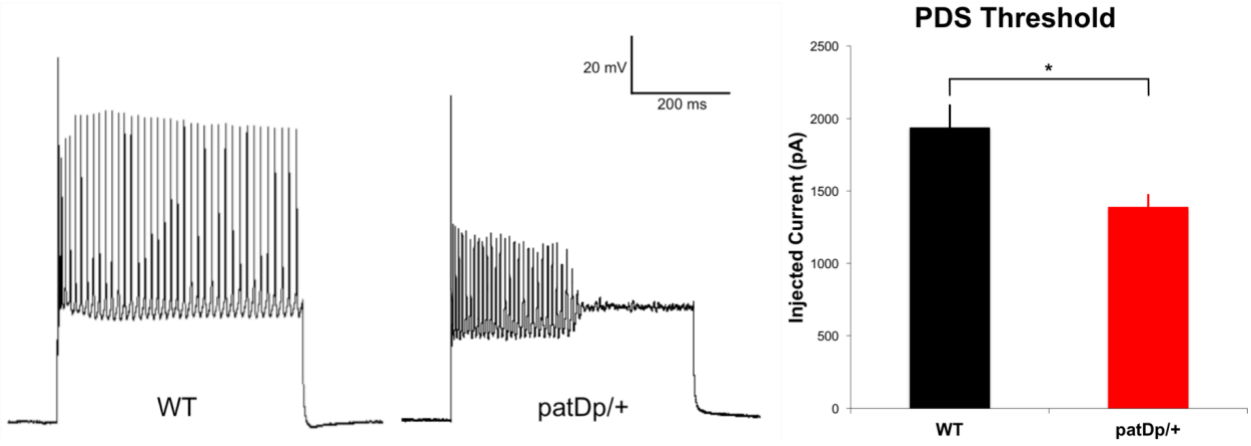

Figure 14: Threshold for PDS is lower in patDp/+ cells. On the left are example traces of cells injected with 1500 pA of current. The WT cell on the far left is able to spike throughout the injection, while the patDp/+ cell has undergone PDS and cannot follow anymore. The bar graph on the right is a quantification of all cells in each group (WT n=19, patDp/+ n=12).

Future Directions

Currently, no seizures have been observed in these mice. However, this may not be as damaging as it initially appears to our experiments. Under the assumption that these mice do in fact have seizures, we would have no idea how frequent these seizures occur. It is possible that these mice have seizures, on average, once a month. There is no current data surrounding the relative levels of severity of seizures in the Dup15q population, so we do not have estimates on how frequently a mouse model of Dup15q would suffer seizures. Epilepsy can sometimes manifest itself in frequent seizures every day, or often can just be a single seizure every couple of months (like my canine companion with epilepsy, Zoe, has). So, given an assumption that these seizures occur once a month and last, at the longest, 10 minutes, it could be very tough to observe one of these seizures. It is however safe to rule out that these mice have seizures multiple times a day, given that this likely would have been witnessed through normal interaction with these mice. But even in the absence of seizures, the difference in PDS could still be relevant and manifest as a reduced threshold to induced seizures.

Another confounding factor in potentially missing these seizures is that seizures can often occur during 'transitional' periods of brain activity, such as waking up from sleep. That could mean that if these mice are in fact seizing, they might be doing so at 7 PM when the facility switches from light to dark, and nobody would be present to observe them at that time of day. To attempt to circumvent some of these issues, we have set up a video camera that observes these mice for 24-hour periods. Having begun some pilot studies of these mice, no seizures have yet been seen during 2 separate 24-hour windows monitoring 3 mice at a time. This camera setup could help with any seizure activity that occurs at times of day without close monitoring, however it doesn't necessarily improve the 'odds' of catching infrequent seizures. One potential

method of capturing differences in propensity to seizure is inducing seizures and looking for threshold of induction/latency to seizure start. This could be much more straightforward to test, although it would completely eliminate any information relating to whether or not patDp/+ mice have spontaneous seizures. A method of testing this begins by injecting the chemoconvulsant pentylenetetrazol and monitoring for seizures (Giardina & Gasior 2009). One potential method is injecting a dose sufficient to cause seizures into mice and timing the latency to seizure initiation. A lower time would indicate that a group (in this case, our patDp/+ mice) were prone to seizures. Secondarily, a threshold could be calculated using the 'up and down' method of induction. In this example, the first mouse from a group is given a sub-threshold injection of a chemoconvulsant. Following this, the dosage is increased in each subsequent mouse until a seizure begins. After that, the next mouse is injected with a lower dosage, such that if a mouse seizes the next dosage is reduced, or if the mouse doesn't seize the next dosage in increased. By averaging the injections in all the mice across the group, a measure of a threshold dosage can be calculated. Comparing thresholds across groups could give a metric of how prone a particular group is to seizures.

Another method that could be helpful is examining EEG signals from each of these mice. In addition to 24-hour camera monitoring, mice can have EEG electrodes implanted and signal could be monitored during the duration of the experiment. Afterwards, EEG signal can be combed through in order to potentially find incidences of seizure or sub-threshold seizure activity. A confound of this experiment is that mice necessarily must have seizure activity in order to find a result, as an increased threshold to seizure is not detectible under these spontaneous conditions. There are pros and cons to this with regards to validating patDp/+ mice as a model of Dup15q. The most 'ideal' result to validate the model would be mice having

spontaneous seizures at a rate similar to humans. However, if this is not the case, it doesn't mean that the model is useless. It might be true that the model only partially recreates a phenotype, which still has the potentially to offer feedback and insights on the human condition. This would therefore make EEG a risky experiment, as the likelihood of a result is lower but the outcome is potentially more impactful. On the other hand, chemoconvulsant threshold tests are very quick and easy, but they lose out on the potential for identifying spontaneous seizures. They would just give a measure of threshold for seizure induction, which could nonetheless prove useful as outlined above.

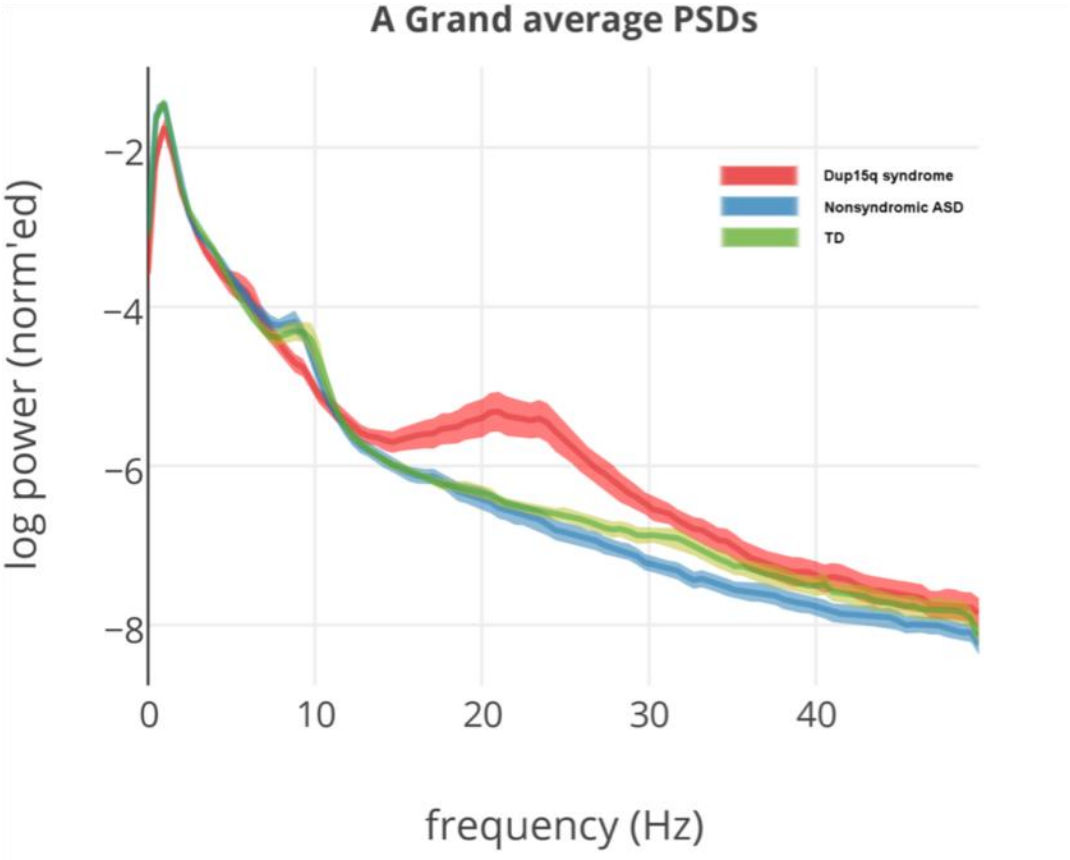

Figure 15: EEG Power Analysis in Dup15q. Figure is from Frohlich et al. 2016, and is data collected from humans. They sought to compare EEG signals from Typical Developing, ASD, and Dup15q children, and found an increase in the beta frequency range.

Luckily there is potentially another option, as EEG recordings in awake mice have begun. As of July 29, 2019, there is a single patDp/+ mouse with an EEG electrode implanted that is generating data for further analysis. This pilot experiment has so far not yielded any positive seizure data, and this mouse might not ever. If seizures are not generalized but focal, placement of the electrode into the seizing area is necessary and might be missed due to our surgical placement being elsewhere (which is initially above somatosensory cortex). However, all is not lost if seizures are not detected. Frohlich et al. in 2016 tested humans using EEG in 3 separate groups: typical development, ASD, and Dup15q. When running a power analysis (which looks at the contribution of particular frequencies to the observed EEG signal), they found that ASD and typical development EEG signals looked very similarly. However, when examining the Dup15q they found an increase in power in the Beta frequency range outlined in Figure 15 (roughly 15-25 Hz). Decreased power in the Beta range has been associated with an increase in inhibition, so increased power led the researchers to conclude that these results are consistent with a decrease in inhibitory signaling. Caveats abound, because likely these Dup15q patients had epilepsy in some form which could be explained by this Beta power result. However, their seizures could actually be causing increased Beta power by modifying transmission, and this is a side effect unimportant to seizure generation (or a side effect of medication). Regardless, the result they found robustly showed that the EEG signals in Dup15q were different even from ASD more broadly.

Outside of looking for seizure activity in our mice, we may also look for a "Dup15q-like" power-signature in the EEG signal from patDp/+ mice. This could allow us to draw additional connections between Dup15q and the mouse model, even if seizures could not be found. And ultimately, the seizure testing now serves two separate functions. The first is to validate the mice

as a good stand-in for Dup15q, allowing much more directed and controlled experiments to understand the condition. Showing seizure activity, which would model the human form of Dup15q, alongside the previously tested 'social deficits', which can be difficult to model in mice, we could offer a great increase in support for the model. But another reason in showing seizures or a propensity to seize would be valuable is in validating PDS as a metric. Testing for PDS is still fairly new, but having a validated cellular correlate of seizures could be great for showing that the patDp/+ model works. However, more importantly is that it could be used elsewhere to test for neural correlates of seizures without needing to do the (previously outlined) difficult and time-consuming work of identifying and classifying seizures.

Continuing to connect and characterize patDp/+ mice in relationship to Dup15q could be very useful in furthering our understanding of the condition. So far, some of the social/communicative deficits have been observed in these mice, and we were able to identify a cellular correlate to the seizure activity seen in humans. Ideally a neural abnormality could be identified that might shed light on a common mechanism between ASD and epilepsy, given the large overlap that exists. This could potentially allow some of these results to not be generalized across different forms of epilepsy, but forms of ASD as well.

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