# A Role for Astrocytes in Nociception, Nucleus Accumbens Physiology, and REM Sleep Regulation

A thesis

submitted by

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**Abstract**

Astrocytes are non-neuronal cells in the nervous system that elevate their intracellular calcium in response to stimuli. Astrocytes express receptors for neurotransmitters and ensheath pre- and post-synaptic neuronal processes. Abundant evidence suggests astrocytes not only detect activity of the synapse, but also release chemicals, termed gliotransmitters, that influence synaptic strength and plasticity. Due to their ability to influence synaptic physiology, astrocytes have gained recognition as modulators of behavior. A very interesting feature of astrocytes is that they are not uniform across the brain. Astrocytes in the spinal cord, for example, have distinct protein expression profiles from those in the nucleus accumbens and the cortex. Moreover, astrocytes have different targets depending on the receptors expressed by surrounding neurons, which are also heterogeneous across brain regions. In this thesis, three distinct roles for astrocytes will be explored: the role of astrocyte gliotransmission in modulating baseline mechanical nociception and nucleus accumbens physiology as well as how astrocyte $IP_3/Ca^{2+}$ signaling regulates REM sleep. Astrocytes release ATP, which is degraded to adenosine that tonically activates adenosine 1 receptors in the hippocampus. Here, adenosine 1 receptors are the dominant adenosine receptor type and through the activation of these receptors, astrocytes increase post-synaptic glutamate receptor function. However in studies conducted in this thesis, decreased A1 receptor activation was not correlated with decreased glutamate receptor function. In the nucleus accumbens, expression levels of adenosine 2 receptors are predominant. This may explain why the gliotransmitter, adenosine does not play the same role in the nucleus accumbens as it does in the hippocampus. In the spinal cord, in addition to adenosine receptors, ATP receptors are prominently expressed. Astrocytes in the dorsal horn of the spinal cord

become reactive after peripheral nerve injury. It is thought that purinergic signaling in the spinal cord mediates persistent nociception. In a mouse model of attenuated gliotransmission, known to have reduced adenosine tone in the cortex and hippocampus, baseline mechanical nociception thresholds are reduced, suggesting gliotransmission has an anti-nociceptive effect. Finally, in the context of sleep, when gliotransmission is attenuated, NREM slow wave activity in the brain is reduced. Interestingly, this phenotype was not recapitulated when astrocyte $IP_3$-mediated $Ca^{2+}$ signaling, thought to be the primary mechanism of calcium signaling, leading to gliotransmission, was attenuated. However, REM sleep was enhanced in these mice. These finding suggest that astrocytes regulate different aspects of sleep through non-overlapping signaling cascades. Overall, this thesis highlights the different functions of astrocytes across regions of the central nervous system.

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**A1R:** Adenosine A1 receptor

**A2AR:** Adenosine A2A receptor

**AMPA:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

**AP:** Action Potential

**ATP:** Adenosine tri-phosphate

**BDNF:** brain derived neurotrophic factor

**BF:** basal forebrain

**$Ca^{2+}$:** Calcium

**cAMP:** cyclic adenosine mono-phosphate

**CCPA:** A1R agonist, 2-chloror-N6 cyclopentyladenosine

**CICR:** Calcium induced calcium release

**CPP:** Conditioned Place Preference

**CPT:** A1R antagonist, 8-cyclopentyltheophyline

**dnSNARE:** transgenic mouse expressing dominant negative SNARE mutation in

**dpi:** days post injury

**EEG:** Electroencephalogram

**EM:** electron microscopy

**EMG:** Electromyogram

**EPSC:** Excitatory Post Synaptic Current

**EPSP:** excitatory postsynaptic potential

**ER:** Endoplasmic Reticulum

**fEPSP:** field excitatory postsynaptic potential

**GABA:** Gamma-Aminobutyric acid

**GFAP:** glial fibrillary acidic protein

**GPCR:** G-Protein Coupled Receptor

**HEK:** human embryonic kidney

**Hz:** Hertz

**i.p.:** intraperitoneal

**$IP_3$:** Inositol triphosphate

**$K^+$:** potassium

**LDCG:** large dense core granule

**lf-SWA:** low frequency SWA

**LTD:** Long Term Depression

**LTP:** Long Term Potentiation

**mEPSC:** miniature Excitatory Post Synaptic Current

**mGluR:** metabotropic glutamate receptor

**MSN:** Medium Spiny Neuron

**NAc:** Nucleus Accumbens

**NMDA:** N-methyl D-aspartate

**NPP:** Neuropathic Pain

**NREM:** Non REM Sleep

**PAP:** perisynaptic astrocyte process

**PFC:** Prefrontal Cortex

**REM:** Rapid Eye Movement Sleep

**SB2:** Synaptobrevin 2

**SLMV:** small synaptic like micro-vesicle

**SWA:** Slow Wave Activity

**Syt:** synaptotagmin

**TenT:** tetanus neurotoxin

**TRPA1:** transient receptor potential A1

**TTX:** Tetrodotoxin

**VAMP:** vesicle-associated membrane protein

**VGCC:** voltage-gated calcium channel

**VGLUT:** vesicular glutamate transporter

**VIPP:** transgenic mouse over-expressing Venus tagged $IP_3$5' phosphatase specifically in astrocytes

**VLPO:** ventrolateral preoptic nucleus

**VTA:** Ventral Tegmental Area

# Chapter 1 Introduction

## Section 1.1 General background:

The history of glial cells began in 1856, when they were named by the German anatomist Rudlof Virchow as “nevernkitt” or nerve-glue. Later in the 1800s and in the early 1900s, Golgi and Cajal made detailed structural observations regarding the glial cell subtype, the astrocyte, and the intimate relationship of astrocytes with neurons and the brain vasculature. Astrocytes have been correlated with higher order neuronal function, as the prevalence of this cell type in the brain has increased throughout evolution, with humans having the highest ratio of astrocytes to neurons.

Today, most studies of astrocyte and neuron function are conducted in the hippocampus, the brain region thought to be involved in declarative memory formation. This region is highly organized structurally and functionally and we understand it best of all areas of the brain. It contains the dentate gyrus, full of excitatory granule cells that project their mossy fibers to area CA3 pyramidal neurons, which in turn send excitatory Schaffer collateral fibers to CA1 pyramidal neuron cell bodies and dendrites.. Also within each of these regions inhibitory interneurons and astrocytes are capable of modulating excitatory transmission. The CA3 to CA1 projection is the most widely studied and understood synapse in the brain, and because of this, it is ideal for testing hypotheses about particular cell types and their functions. Many studies reviewed here have done just that.

The notion that glial cells modulate synapse morphology, function, and plasticity can no longer be introduced as a recent finding or a new idea. Over twenty years ago, astrocytes were found to be involved in the formation of synapses (Palacios-Pru, Palacios

et al. 1979). From that point on, the investigation of astrocytes followed a similar progression to that of neurons, starting with findings in dissociated culture that astrocytes express receptors for major neurotransmitters such as GABA and glutamate. Early studies of astrocytes also revealed that astrocytes release chemicals that influence neurons. These chemicals, termed gliotransmitters, act on neuronal receptors to influence their function. The discovery that there is cross talk between astrocytes and neurons at the synapse paved the way for all future studies on the tripartite synapse. This section will provide the reader with a review of the findings that pertain to the role of astrocytes in the tripartite synapse.

**Section 1.2 Structure implies function:**

Most introductions to a biological element begin with a description of its structure, progress to what function that structure implies and finally provide the evidence that supports that function. Astrocytes are abundant in the human brain. Astrocytes consist of a central cell body and highly ramified processes that extend to neurons, other glia, and the vasculature. Astrocytes occupy non-overlapping domains (~100μm in diameter), each encompassing hundreds of thousands of synapses (Bushong, Martone et al. 2002, Halassa, Fellin et al. 2007). Although astrocytes occupy distinct domains, they are interconnected with each other via gap junctions, which allow passage of electrical and chemical signals. The structures of astrocytes and their coverage of synapses vary across brain regions: the rate of synaptic ensheathment is estimated at about 50% in the cortex and hippocampus, but reaches 80-90% in the cerebellum (Lippman, Lordkipanidze et al. 2008). Furthermore, electron microscopy evidence suggests astrocytic coverage of synapses varies within brain regions from no contact at

all to minimal contact and full synaptic ensheathment (Lehre and Danbolt 1998, Rusakov and Kullmann 1998, Rusakov and Kullmann 1998, Rusakov and Kullmann 1998, Ventura and Harris 1999). It is thought that the level of synaptic coverage by perisynaptic astrocyte processes (PAPs) is activity dependent, where more highly active synapses have increased coverage (Ventura and Harris, 1999). There is also some evidence that astrocyte morphology in mice changes in response to enriched environmental conditions (Soffie, Hahn et al. 1999). Given the sensitivity of astrocytes to input within the brain and to behavioral manipulations, they must express some sort of detection mechanism.

**Section 1.3 Astrocytes Detect Neuronal Activity:**

Astrocytes are able to sense changes in the surrounding neuropil, evidenced by their expression of a plethora of excitatory, inhibitory, and modulatory ionotropic (Martin, Blackstone et al. 1993, Fraser, Duffy et al. 1995) and metabotropic (Cai, Schools et al. 2000, Araque, Martin et al. 2002) neurotransmitter receptors. Astrocytes also express receptors for growth factors (Liu and Neufeld 2007) and peptides. They even express orphan receptors of unknown function (Cahoy, Emery et al. 2008). *Ex vivo* astrocytes throughout the cortex and hippocampus have been shown to respond to glutamate via type 1 and type 2 metabotropic glutamate receptors (mGluRs) (Porter and McCarthy 1996, Pasti, Volterra et al. 1997, Latour, Gee et al. 2001). They are also responsive to norepinephrine, acetylcholine, histamine, adenosine, ATP, GABA and serotonin (Hertz, Chen et al. 2004, Verkhratsky, Rodriguez et al. 2012).

Aside from detection of activity through receptors, astrocytes express amino acid transporters, mainly GLT-1 and GLAST (Gupta and Prasad 2014), allowing uptake of glutamate released by neurons. Such uptake mechanisms are thought to maintain proper

synapse function (Rothstein, Dykes-Hoberg et al. 1996, Furuta, Rothstein et al. 1997, Danbolt 2001, Sattler and Rothstein 2006), as excess extracellular glutamate is toxic to neurons. Astrocytes convert glutamate to glutamine and return this precursor to neurons for future glutamate synthesis. Astrocytes also buffer $K^+$ ions to regulate neuronal excitability (Walz 2000, Wang, Smith et al. 2012). Resting extracellular $K^+$ is maintained at 3 mM; after high levels of neuronal activity, extracellular $K^+$ can rise to as high as 5 mM, sufficient to depolarize neurons to near or above threshold for action potential firing, which can produce epileptiform activity (Walz 2000). Astrocytes also regulate resting levels of $K^+$ via their high expression of inward rectifying $K^+$ channels (Sibille, Pannasch et al. 2014). This high $K^+$ conductance largely explains why astrocytes have such hyperpolarized resting membrane potentials close to the reversal potential of potassium (Butt and Kalsi 2006). Although the mechanisms of $K^+$ and glutamate uptake were initially thought of as passive synaptic maintenance, it is now clear that astrocytes modulate $K^+$ and glutamate transporter expression in an activity-dependent manner that can synchronize neuronal networks within an astrocytic domain and can tune the excitability of neuronal networks by altering their firing thresholds. The next sub-section will address the receptor- and transporter- mediated signaling mechanisms in astrocytes.

**Section 1.4 $Ca^{2+}$ signaling in astrocytes:**

As astrocytes are equipped with many ways to respond to stimuli, this section will review the downstream signaling after receptor activation. The major mechanism of astrocyte signal transduction is intracellular $Ca^{2+}$ release. The signals that have been shown to activate astrocyte receptors linked to calcium signaling are norepinephrine (Duffy and MacVicar 1995), glutamate (Porter and McCarthy 1995, Porter and McCarthy

1996, Pasti, Volterra et al. 1997), GABA (Kang, Jiang et al. 1998), acetylcholine (Araque, Martin et al. 2002), histamine (Shelton and McCarthy 2000), adenosine (Porter and McCarthy 1995), and ATP (Bowser and Khakh 2007). This list is ever growing; however, the rigor at which new studies are expected to test hypotheses is slowing progress considerably. Recent work largely focuses on $Ca^{2+}$ imaging in awake, behaving mice, as slice preparations and anesthesia are both known to affect intracellular $Ca^{2+}$ dynamics (Thrane, Rangroo Thrane et al. 2012). Even still, many methods of imaging $Ca^{2+}$ are still heavily criticized (Volterra, Liaudet et al. 2014), as $Ca^{2+}$ indicators act as chelators, altering $Ca^{2+}$ signaling and homeostasis. Although some may discount *in situ* experiments, many early labs investigating $Ca^{2+}$ signaling made discoveries based on elegantly designed and controlled studies.

Although electrically inexcitable due to insufficient (D'Ascenzo, Vairano et al. 2004) voltage sensitive ion channel expression, astrocytes are capable of elevating their internal $Ca^{2+}$ concentration in response to external stimuli (Porter and McCarthy 1995, Porter and McCarthy 1996). The dynamics of such changes in $Ca^{2+}$ concentration range from rapid transient elevations ("spikes") to extended "plateaus." As in neurons, $Ca^{2+}$ signals can be spatially restricted in astrocytes, confined to micro (or nano) domains within astrocytes (Pasti, Volterra et al. 1997, Grosche, Matyash et al. 1999, Grosche, Kettenmann et al. 2002); they can also occur more globally, across the astrocyte (Carmignoto 2000, Carmignoto 2000, Araque, Martin et al. 2002) and even spread as waves between astrocytes (Arcuino, Lin et al. 2002). Inter–astrocyte propagating $Ca^{2+}$ waves involve ATP release from one astrocyte activating plasma membrane ATP receptors on neighboring astrocytes (Guthrie, Knappenberger et al. 1999). There is also

evidence that $Ca^{2+}$ waves are propagated by inositol triphosphate ($IP_3$) and $Ca^{2+}$ ions flowing between astrocytes via large conductance gap junctions (Leybaert, Paemeleire et al. 1998). In the hippocampus $Ca^{2+}$ "oscillations" can be intrinsically generated independent of neuronal activity (Nett, Oloff et al. 2002). Astrocytes may govern discrete nodes of activity that may be coupled through gap junctions or inter-astrocyte $Ca^{2+}$ signaling (Halassa, Fellin et al. 2007). Such $Ca^{2+}$ signals are complex, and which types of signal exist *in vivo* under physiological conditions is a heavily debated topic. Furthermore, the magnitude, spatial, and temporal resolution of the detected $Ca^{2+}$ signal may depend on the experimental preparation as well as the tools used for measurement. Finally, there is no general consensus in the field as to what to call each variation of a $Ca^{2+}$ signal (Volterra, Liaudet et al. 2014). For these reasons, I will generically refer to a $Ca^{2+}$ response as a signal, and I will mainly focus on the molecular signaling cascade and the physiological events leading up to and following the $Ca^{2+}$ signal, rather than the details of the signal itself.

$Ca^{2+}$ signals, as we understand them today, are largely produced by receptor-mediated release from internal stores. Early studies found that depleting extracellular $Ca^{2+}$ did not prevent glutamate-induced astrocytic $Ca^{2+}$ elevation (Charles, Merrill et al. 1991). Furthermore, inhibition of the endoplasmic reticulum (ER) pump with thapsagargin prevented ATP-evoked $Ca^{2+}$ signals from occurring (Centemeri, Bolego et al. 1997). The most widely studied molecular pathway of ER-mediated $Ca^{2+}$ release is $IP_3$ receptor-dependent release from internal stores. There are three different $IP_3$ receptors, 1, 2, and 3. Astrocytes express type 2 $IP_3$ receptors and smooth ER has been identified in PAPs (Spacek and Harris 1998, Holtzclaw, Pandhit et al. 2002, Bezzi, Gundersen et al.

2004). The canonical mechanism by which $IP_3$ receptor mediated release from the ER occurs is via activation of metabotropic receptors coupled to phospholipase C (Venance, Stella et al. 1997). Mechanisms of $IP_3/Ca^{2+}$ signaling in astrocytes have been linked to Group I mGluR (mGluR types 1 and 5) activation (Floyd, Rzigalinski et al. 2004).

Although less extensively studied, there are many alternative mechanisms that mediate $Ca^{2+}$ dynamics in astrocytes. A less common mechanism of release from internal stores is via activation of ryanodine receptors, which are sensitive to caffeine and mediate calcium induced calcium release (CICR; (Reyes and Parpura 2009, Parpura, Grubisic et al. 2011). Astrocytes also possess the means to permit $Ca^{2+}$ entry from extracellular stores due to their expression, albeit low, of voltage-gated $Ca^{2+}$ channels, ionotropic receptors, and capacitative calcium entry (Verkhratsky and Kettenmann 1996). Functional data suggest that sustained $Ca^{2+}$ responses in culture require plasma membrane influx of $Ca^{2+}$ ions, possibly through $Ca^{2+}$-permeant kainate receptors (Cornell-Bell, Finkbeiner et al. 1990). Very recent data suggest that astrocytes permit extracellular $Ca^{2+}$ entry through transient receptor potential A1 (TRPA1) channels (Shigetomi, Tong et al. 2012, Shigetomi, Jackson-Weaver et al. 2013, Tong, Shigetomi et al. 2013). TRPA1 channels operate independently of internal $Ca^{2+}$ stores and are a membrane-specific mechanism of intracellular $Ca^{2+}$ elevation.

Many of the findings regarding $Ca^{2+}$ signaling in astrocytes were conducted in dissociated culture or acute slices. However, work by Hirase et al., 2004 revealed spontaneous and activity-dependent calcium transients in astrocytes, visualized through cranial windows in live rats (Hirase, Qian et al. 2004). They also confirmed that astrocyte $Ca^{2+}$ signaling is positively correlated with increased neuronal activity (Hirase, Qian et al.

2004). Additionally, $Ca^{2+}$ elevations have been found *in vivo* in response to physiological stimuli, such as whisker stimulation in mice (Gurden, Uchida et al. 2006, Wang, Zhou et al. 2006, Schummers, Yu et al. 2008, Lind, Brazhe et al. 2013). $Ca^{2+}$ signals are thought to be restricted to single astrocytes in vivo (Wang, Zhou et al. 2006), but it should be noted that anesthesia affects $Ca^{2+}$ signaling in astrocytes (Thrane, Rangroo Thrane et al. 2012). It is therefore important that experiments have confirmed that astrocytes respond to physiological stimuli in awake, behaving mice (Dombeck, Khabbaz et al. 2007, Hoogland, Kuhn et al. 2009, Nimmerjahn, Mukamel et al. 2009).

A key issue regarding $Ca^{2+}$ signaling in astrocytes is whether it serves to integrate input signals to allow for plastic changes and regulated output signals that modulate synaptic function (Pasti, Volterra et al. 1997). Astrocytes in the hippocampus are able to distinguish between and non-linearly integrate inputs from glutamatergic schaffer collateral and glutamatergic/cholinergic alveus (Araque, Martin et al. 2002, Perea and Araque 2005). Furthermore, $Ca^{2+}$ signaling has been shown to influence neuronal gene expression in a frequency-dependent manner (West, Chen et al. 2001). It is highly likely that astrocytes also respond differently to varying $Ca^{2+}$ signals. Indeed, there is evidence in support of this: $Ca^{2+}$ oscillations regulate pulsating glutamate release, but sustained $Ca^{2+}$ elevations only cause a brief release of glutamate at the onset of the rise in $Ca^{2+}$ (Pasti, Zonta et al. 2001). Also glutamate release may occur specifically at the rising phase of intracellular $Ca^{2+}$ in astrocytes (Perea and Araque 2005). Furthermore, the degree of astrocyte activation corresponds to the surrounding active synapses (Honsek, Walz et al. 2012). The prevailing hypothesis is that $IP_3$ signaling mediates larger scale $Ca^{2+}$ responses that may encompass an entire astrocyte but local $Ca^{2+}$ transients at the cell

membrane, and possibly around synapses, may be independent of $IP_3$ signaling. Thus, there are two known mechanisms of $Ca^{2+}$ signaling in astrocytes that may act in a mutually exclusive manner (although it has been hypothesized that sub-threshold $Ca^{2+}$ transients can result in larger $IP_3$ driven signals). Finally, release of various gliotransmitters may be regulated by different $Ca^{2+}$-dependent mechanisms. Glutamate release is consistently tied to $IP_3$-mediated $Ca^{2+}$ signaling. However, D-serine requires CICR and extracellular $Ca^{2+}$ entry through TRPA1 channels (Mothet, Pollegioni et al. 2005, Shigetomi, Bushong et al. 2013).

Now that I have reviewed the activation of astrocytes and how they integrate information via different mechanisms of $Ca^{2+}$ signaling, I will move on to the various ways astrocytes can influence their surroundings through release of neuroactive and vasoactive agents, through coverage of synapses, and through regulating ion and neuroactive substance concentrations in the synaptic cleft. Through these mechanisms, astrocytes are capable of influencing synaptic function and plasticity, and ultimately animal behavior. Naturally, I will begin with the structural influence astrocytes have over their surroundings.

**Section 1.5 Astrocytes influence brain function**

Synaptic coverage by peripheral astrocyte processes is plastic and responsive to activity dependent changes in glutamate (Theodosis and Poulain 1999, Oliet, Piet et al. 2001, Genoud, Quairiaux et al. 2006, Lavialle, Aumann et al. 2011, Bernardinelli, Muller et al. 2014, Bernardinelli, Randall et al. 2014). PAPs are capable of reversibly remodeling their synaptic coverage in a matter of minutes to hours. In the hypothalamus, astrocyte structural plasticity follows a circadian rhythm (Girardet, Becquet et al. 2010). Structural

plasticity in hippocampal astrocytes has also been demonstrated *in situ*, where astrocyte PAP motility is coordinated with dendritic spine maturity (Haber, Zhou et al. 2006) and modifies synaptic connectivity (Verbich, Prenosil et al. 2012). These studies provide evidence for a role for astrocytes in structural plasticity of the synapse.

Early discoveries of astrocytes highlighted their functional role in metabolism due to their intimate relationship with both neurons and the vasculature. Indeed, astrocytes play a role in metabolic coupling between capillaries and neurons (Pellerin and Magistretti 1994, Fray, Forsyth et al. 1996, Hu and Wilson 1997, Hu and Wilson 1997, Vesce, Bezzi et al. 1999, Pellerin and Magistretti 2003, Pellerin and Magistretti 2003, Gordon, Choi et al. 2008, Allen and Barres 2009, Kasischke 2011). Astrocytes are able to take up pyruvate from neurons as well as glucose from capillaries and shuttle lactate as a nutrient back to neurons (Kasischke 2011). This process is tied to the uptake of glutamate from the synaptic cleft, which is primarily done by astrocytes via the glutamate transporters GLT-1 and GLAST (Armbruster, Hampton et al. 2014). When glutamate is taken up by astrocytes, $Na^+$ enters the cell and the $Na^+/K^+$ ATPase is activated. This creates a demand for glucose that is then taken by astrocytes from the capillaries. In this way, astrocytes react to neuronal activity by responding to release of the neurotransmitter glutamate and subsequently providing energy in the form of lactate back to the neurons when they need it most. Astrocytes promote vasodilation via $Ca^{2+}$ dependent release of vasodilators such as cyclooxygenase and prostaglandin (Bezzi, Carmignoto et al. 1998, Zonta, Angulo et al. 2003, Zonta, Sebelin et al. 2003), the first of many substances found to be released by astrocytes that will be discussed in this introduction.

It is widely accepted that astrocytes express receptors sensitive to neurotransmitters and respond to receptor activation with increases in intracellular $Ca^{2+}$, but the extent to which these $Ca^{2+}$ signals provide physiological output from astrocytes, e.g. gliotransmission, is still under debate. Despite this controversy, there is substantial evidence that astrocytes detect input and modulate their output via local $Ca^{2+}$ signaling around synapses (Di Castro, Chuquet et al. 2011). $Ca^{2+}$ signaling leads to release of gliotransmitters, defined as substances that are synthesized and/or stored in astrocytes, that are released in response to stimuli, that initiate responses in neighboring cells, and that play a role in regulating activity (Parpura and Haydon 2000). There are three main classes of gliotransmitters: amino acids such as glutamate and D-serine, nucleotides like adenosine 5'-triphosphate (ATP), and peptides, namely brain-derived neurotrophic factor (BDNF). Astrocytes may release gliotransmitters through a variety of transporters, hemichannels, or vesicles. These mechanisms will now be described.

Vesicular-mediated release is the most studied form of gliotransmission as it is most likely to occur under physiological conditions. There is extensive evidence that astrocytes are able to release gliotransmitters in response to neuronal activity. One way gliotransmission occurs is via a $Ca^{2+}$- and SNARE- (Soluble N-ethyl-maleimide-sensitive fusion protein Attachment protein REceptor) dependent exocytosis of vesicles (Hua and Scheller 2001). To form a functional SNARE complex in neurons, VAMP2, SNAP25, and STX1 bind together (Sudhof and Rothman 2009). These proteins have many isomers; SNAP23, VAMP3, and STX1 are all expressed in PAPs (Schubert, Bouvier et al. 2011). Furthermore, astrocytes express the SNARE associated Synaptotagmin 4 (Syt4) as a $Ca^{2+}$ sensor for vesicle release (Zhang, Fukuda et al. 2004). The first evidence that $Ca^{2+}$

dependent transmitter release occurred in astrocytes was obtained from application of $Ca^{2+}$ ionophore in culture. Parpura et al. (1994) showed that elevated intracellular $Ca^{2+}$ was necessary and sufficient to induce glutamate release from astrocytes in culture (Parpura, Basarsky et al. 1994). Many experimental results have suggested that astrocytes release vesicles. Increasing astrocyte $Ca^{2+}$ increases membrane capacitance, suggestive of vesicular fusion with the plasma membrane (Kreft, Stenovec et al. 2004, Zhang, Pangrsic et al. 2004). Two forms of vesicular fusion are supported in astrocytes, kiss and run, and full fusion. The specific Syt for each type (Syt4 for kiss and run and Syt1 for full fusion) are both expressed in astrocytes (Bezzi, Gundersen et al. 2004, Kreft, Stenovec et al. 2004, Zhang, Pangrsic et al. 2004, Chen, Wang et al. 2005, Crippa, Schenk et al. 2006). Structural evidence also supports both types of vesicle expression and plasma membrane fusion, shown by omega shaped vesicles along the plasma membrane (Coco, Calegari et al. 2003, Montana, Malarkey et al. 2006).

Astrocytes contain secretory organelles including small synaptic like microvesicles (SLMV) that are expressed in PAPs (Bezzi, Gundersen et al. 2004, Jourdain, Bergersen et al. 2007, Bergersen and Gundersen 2009). Large dense-core granules (LDCGs) are also expressed in astrocytes (Coco, Calegari et al. 2003, Ramamoorthy and Whim 2008, Prada, Marchaland et al. 2011). Astrocytes may also store and release adenosine and ATP from lysosomes (Bowser and Khakh 2007). Synaptobrevin 2 (Sb2) is one molecule in the SNARE complex that is expressed on both small and large vesicles. EM evidence from cultured astrocytes demonstrates vesicles, some of which are fused with the membrane (Coco, Calegari et al. 2003, Montana, Malarkey et al. 2006). The smaller vesicles contain D-serine (Mothet, Pollegioni et al.

2005) and glutamate, indicated by their coexpression of VGLUTs 1 and 2 (Bezzi, Gundersen et al. 2004, Bergersen, Morland et al. 2012, Martineau 2013, Martineau, Shi et al. 2013) and the larger, dense core vesicles contain ATP (Coco, Calegari et al. 2003). Chen et al., 2005 also demonstrated dye-filled vesicles in astrocytes fused with the plasma membrane (Chen, Wang et al. 2005). Furthermore, biosensor sniffer HEK (human embryonic kidney) cells expressing NMDAR (to detect glutamate) responded to $Ca^{2+}$ elevations in astrocytes (Pasti, Zonta et al. 2001). The biosensor cells detected no release in the presence of tetanus neurotoxin (TenT) to inactivate VAMP2/3 (another name for Sb2/3) and disrupt vesicular fusion of the SNARE complex and bafilomycin $A_1$ to inhibit the vacuolar-type $H^+$-ATPase, preventing transmitter loading into vesicles. Furthermore, other groups have shown that exocytosis is blocked by infusion of TenT in astrocytes (Jourdain, Bergersen et al. 2007, Perea and Araque 2007). In summary, these data support that astrocytes express functional machinery for $Ca^{2+}$-dependent vesicular SNARE-mediated release.

There is evidence of expression of the transporters necessary for loading of gliotransmitters into vesicles. For glutamate, VGLUT1 or 2 (Bezzi, Gundersen et al. 2004, Zhang, Fukuda et al. 2004) and for ATP, vesicular nucleotide transporter (VNUT) expression has been found (Sawada, Echigo et al. 2008). Inhibition of VGLUT with Rose Bengal resulted in decreased $Ca^{2+}$-dependent glutamate release from astrocytes (Montana, Ni et al. 2004). Genetic manipulation of the SNARE protein, Sb2, and RNA interference with the $Ca^{2+}$ binding domain of the SNARE complex, Syt IV, both inhibited glutamate release from astrocytes (Zhang, Fukuda et al. 2004, Zhang, Pangrsic et al. 2004). Gliotransmitter containing vesicles and glutamate itself are immobilized and

enriched at he end of astrocyte processes (Anlauf and Derouiche 2005, Crippa, Schenk et al. 2006). Finally, EM evidence shows PAPs contain ER (Spacek and Harris 1998, Bezzi, Gundersen et al. 2004) and $IP_3R2$ immunoreactivity (Holtzclaw, Pandhit et al. 2002) suggestive of an organized domain for activation of release of vesicles.

Due to the similarity to neuronal mechanisms and the largest potential for a role in physiologically relevant phenomena, vesicular mediated release is the most widely studied mechanism of gliotransmission. However, astrocytes are also capable of gliotransmission via anion transporters and connexin hemichannels (Montero and Orellana 2014). There is additional evidence that astrocytes can release gliotransmitters through P2X7 receptor channels (Kukley, Barden et al. 2001, Duan, Anderson et al. 2003), by $Ca^{2+}$-exchange through the cysteine-glutamate transporter (Tang and Kalivas 2003), and through various other channels under pathological conditions (Santello, Cali et al. 2012).

Connexins form gap junctions between cells, but form hemichannels when they are expressed on a free membrane exposed to the extracellular space (Herve and Derangeon 2013). Another pore-forming protein family, pannexins, also form hemichannels in astrocytes (Montero and Orellana 2014). Due to their large pore diameter, hemichannels are able to open and release glutamate, ATP, and other small molecules (Kang, Kang et al. 2008).

Glutamate is the most widely studied and accepted gliotransmitter. It is synthesized in astrocytes (Hertz, Dringen et al. 1999), transported into vesicles that fuse with the plasma membrane (Montana, Ni et al. 2004, Crippa, Schenk et al. 2006) that permit quantal release (Pasti, Zonta et al. 2001) in a SNARE- and $Ca^{2+}$-dependent

mechanism (Parpura, Basarsky et al. 1994, Araque, Li et al. 2000, Hua, Malarkey et al. 2004, Kreft, Stenovec et al. 2004, Zhang, Fukuda et al. 2004, Zhang, Pangrsic et al. 2004, Pascual, Casper et al. 2005). Such gliotransmission typically results from increased $IP_3$ signaling from type 1 mGluR stimulation on astrocytes. However, evidence from astrocyte cultures suggests glutamate release may also require CICR via ryanodine sensitive $Ca^{2+}$ stores (Hua, Malarkey et al. 2004). Bezzi et al., 1998 found that prostaglandins can also induce $Ca^{2+}$-dependent glutamate release. Aside from $Ca^{2+}$-dependent release, glutamate can be released by $Ca^{2+}$-independent mechanisms, including reverse operation of glutamate transporters, the cysteine-glutamate antiporter, release through $P2X_7$ receptor pores (Duan, Anderson et al. 2003), swelling-induced operation of anion channels, and via connexin and/or pannexin channels (Parpura, Scemes et al. 2004, Malarkey and Parpura 2008).

The effects of glutamate release from astrocytes are vast. It increases slow inward currents in surrounding neurons via NMDAR activation (Parpura and Haydon 2000, Fellin, Pascual et al. 2004, D'Ascenzo, Fellin et al. 2007) and may locally entrain groups of neurons to fire in synchrony (Fellin, Pascual et al. 2004, Halassa, Fellin et al. 2007). Furthermore, $Ca^{2+}$ dependent release of glutamate from astrocytes enhances release probability from neurons via activation of group I mGluRs or extrasynaptic NMDARs (Araque, Sanzgiri et al. 1998, Jourdain, Bergersen et al. 2007, Perea and Araque 2007, Navarrete, Perea et al. 2012) enabling astrocytes to coordinate neuronal glutamate activity both pre- and post-synaptically.

$Ca^{2+}$ dependent glutamate released from astrocytes affects synaptic strength and plasticity (Jourdain, Bergersen et al. 2007, Santello and Volterra 2009). Depending on

how glutamate release is evoked from astrocytes, the effect on neuronal function varies. Astrocytes mediate heterosynaptic depression in the hippocampus via activation of group II (types 2 and 3) and III (types 4, 6, 7, and 8) mGluRs. Perea and Araque, 2007 demonstrated that astrocytic glutamate activation of mGluRs influences long-term potentiation (LTP). Glutamate released from astrocytes also influences spike timing dependent plasticity through a presynaptic NMDAR mechanism, controlling long-term depression (LTD) (Min and Nevian 2012). Finally, PAR-1 receptor stimulation on astrocytes increased post-synaptic neuronal excitability (Shigetomi, Bowser et al. 2008).

The second most widely studied gliotransmitter is ATP. Unlike glutamate, evidence suggests ATP as a chemical transmitter is primarily derived from astrocytes (Zhang, Wang et al. 2003, Pascual, Casper et al. 2005). Evidence suggests ATP is stored separately from glutamate in dense core vesicles and lysosomes in astrocytes and is released both tonically and phasically in a $Ca^{2+}$- and SNARE-dependent manner (Pascual, Casper et al. 2005). ATP can directly activate purinergic receptors on neurons by activating ionotropic P2X receptors or metabotropic P2Y receptors (Jourdain, Bergersen et al. 2007, Pascual, Ben Achour et al. 2012). However, upon release into the extracellular space, ATP is mostly degraded to adenosine as a result of rapid hydrolysis by ectonucleotidases (Dunwiddie, Diao et al. 1997). Astrocyte derived adenosine acts on presynaptic A1Rs on neurons to tonically inhibit excitatory post-synaptic potentials (EPSPs). Astrocyte activation of A1Rs also mediates heterosynaptic depression in the hippocampus (Pascual, Casper et al. 2005, Halassa, Florian et al. 2009). Additionally, post-synaptic A1R activation initiates a src/fyn kinase cascade to phosphorylate and insert NMDARs into the post synaptic membrane, influencing the capacity for synaptic

plasticity (Zhang, Wang et al. 2003, Pascual, Casper et al. 2005, Martin, Fernandez et al. 2007, Deng, Terunuma et al. 2011, Panatier, Vallee et al. 2011). Astrocytic adenosine can also activate A2ARs to enhance presynaptic release probability or post-synaptic neuronal excitability in regions where this receptor subtype is expressed (Dias, Rombo et al. 2013).

D-serine is a co-agonist for the glycine binding site on NMDARs. Co-activation of glutamate and the co-agonist is required for receptor activation. In the brain, D-serine is produced exclusively by astrocytes and is packaged into SLMVs (Martineau, Shi et al. 2013) and released in response to non-NMDA glutamate receptor stimulation (likely, mGluR activation; (Schell, Molliver et al. 1995). Release of D-serine is sensitive to Concanamycin A and TeNT and is therefore likely to be loaded into vesicles and released via SNARE-mediated vesicular fusion. Furthermore D-serine release requires both intracellular and extracellular $Ca^{2+}$ (Mothet, Pollegioni et al. 2005). Specifically, D-serine release requires a unique $Ca^{2+}$ elevation dependent on TRPA1 receptors on the plasma membrane (Henneberger, Papouin et al. 2010, Henneberger and Rusakov 2010). Astrocyte derived D-serine acts on neuronal NMDARs and modulates their function (Panatier, Theodosis et al. 2006, Oliet and Mothet 2009, Fossat, Turpin et al. 2012). Furthermore, D-serine from astrocytes is required for LTP induction in cortical slices (Fossat, Turpin et al. 2012). In somatosensory and visual cortex, cholinergic activation of astrocytes stimulates the release of D-serine or glutamate, which, in association with whisker (Takata, Mishima et al. 2011) or visual stimuli (Chen, Sugihara et al. 2012) mediate long-term plasticity changes in cortical sensory responses mediated by NMDA receptors.

Many other substances besides glutamate, ATP and D-serine are reportedly released by astrocytes. BDNF is taken up by astrocytes and stored in dense core vesicles in astrocytes and released (Bergami, Santi et al. 2008). Data suggests tumor necrosis factor alpha (TNFα) is also released by astrocytes and increases synaptic strength through insertion of AMPARs (Beattie, Stellwagen et al. 2002, Stellwagen and Malenka 2006).

Despite the mounting evidence that astrocytes release gliotransmitters that affect neuronal activity (Kang, Jiang et al. 1998, Fellin, Pascual et al. 2004, Pascual, Casper et al. 2005, Perea and Araque 2005, Panatier, Theodosis et al. 2006, Stellwagen and Malenka 2006, Perea and Araque 2007, Santello and Volterra 2009, Araque and Navarrete 2010, Henneberger, Papouin et al. 2010, Di Castro, Chuquet et al. 2011, Panatier, Vallee et al. 2011, Fossat, Turpin et al. 2012, Santello, Cali et al. 2012), some groups have failed to reproduce such findings. McCarthy's group used a mouse line expressing an exogenous Gq-coupled MRGA1 receptor specifically in astrocytes and reported that stimulation of these receptors which led to $Ca^{2+}$ increases in astrocytes did not affect synaptic function or LTP (Fiacco, Agulhon et al. 2007, Agulhon, Fiacco et al. 2010). This same group also found that a constitutive knock out of the $IP_3$ receptor, $IP_3R2$ KO mice, didn't have any effect on synaptic transmission or plasticity (Bonder and McCarthy 2014, Petravicz, Boyt et al. 2014). Considering that astrocyte $Ca^{2+}$ signaling is temporally regulated, activation via a receptor that is not endogenously expressed and finding a lack of effect on neuronal activity does not rule out the possibility that physiological $Ca^{2+}$ signaling modulates neuronal function. Furthermore, in the case of the constitutive $IP_3R2$ knock out mouse, many developmental adaptations may take place

when a signaling cascade so important as IP3R2-dependent $Ca^{2+}$ signaling is knocked out. While these studies demonstrate that different methods used from one group to another can yield varying results, they do not shed significant doubt on the role of astrocytic $Ca^{2+}$ as a modulator of neuronal function.

The influence of astrocytes on neuronal network function is undoubtedly complex. It has yet to be determined whether a single astrocyte is capable of releasing multiple gliotransmitters, whether gliotransmitters can be co-released, or whether different gliotransmitters might be released in a stimulus-specific manner. Given that astrocytes are able to distinguish separate inputs (Perea and Araque 2005), it is likely that they allow for information processing for selection of appropriate outputs. Astrocytes have varying levels of coverage around synapses and studies in different brain regions have yielded varying results as far as the influence astrocytes have over synaptic transmission. Therefore, it is likely that the cell type we call an astrocyte is heterogenous across regions and perhaps within regions; thus, conclusions from single brain region studies may not be applicable across the brain. Future studies to determine the heterogeneity of astrocytes probing the same mechanisms in different brain regions will lead to a better understanding of glia in cellular synaptic network function. Until then, genetic manipulations of astrocytes and the assessment of a whole brain or behavioral output are the best way to test a single function in regulating circuit activity and behavior. The following sections will expand on three behaviors to be discussed and provide detailed information regarding specific ways astrocytes are positioned within each system to modulate behavior.

**Section 1.6: Nociception and Gliotransmission**

Pain sensation is an adaptive response to impending tissue damage that protects an organism from extended injury. Acute nociception begins with activation of primary afferent neurons whose cell bodies reside in the trigeminal or dorsal root ganglia (DRG), just outside the spinal cord in the peripheral nervous system (PNS). However, their synaptic terminals end in the dorsal horn of the spinal cord, within the central nervous system (CNS), where second order neurons transmit information to the brain. Three classes of neurons share this location. First, the large diameter, myelinated Aβ fibers with high conduction velocities that relay sub-pain threshold touch signals via glutamate release (Djouhri and Lawson 2004). The second type of neuron in this location, the Aδ type, is intermediate in size, thinly myelinated, and has medium conduction velocities. The third type of neuron in this region is the C fiber type, which is unmyelinated, smaller, and has slow conduction velocities. Nociceptive stimuli activate Aδ and C fiber type neurons, which release the excitatory amino acid, glutamate (Julius and Basbaum 2001). Acute pain is primarily mediated by post-synaptic AMPAR activation. Nociceptive neurons mainly synapse on neurons residing in the first and second lamina of the dorsal horn, and sub-pain threshold neurons synapse on deeper layer five neurons. However, some medium Aδ fibers also synapse on deeper layer five dorsal horn neurons. Painful stimuli cause medium Aδ and C fibers to co-release glutamate and peptides (substance P and calcitonin gene-related peptide, CGRP), which enhances excitatory post-synaptic responses of second order neurons (Ju, Hokfelt et al. 1987, Ju, Melander et al. 1987). High frequency activation of these neurons may result in temporary hypersensitivity of the synapse, called wind-up (Dickenson and Sullivan

1987). This is due to increased excitability of post-synaptic neurons due to sufficient depolarization of second order neurons, allowing for increased conductance through NMDARs. This homosynaptic response is an activity dependent and adaptive mechanism to avoid tissue damage as repeated mild noxious stimuli can become increasingly harmful. Under pathological conditions, the balance between noxious and innocuous stimuli is disrupted and chronic, maladaptive pain can occur. These mechanisms first involve activity dependent integration of physiological stimuli that result in lasting, activity-independent alterations in the sensitivity that spreads beyond the initial site of hyper excitation. The mechanisms by which this occurs are incompletely understood. A major obstacle in elucidating the relative contribution of specific cell types is reflected by the ubiquitous nature of the signaling molecules that have been implicated in the behavioral expression of pain. ATP, adenosine and glutamate are not only ubiquitous regulators of normal nervous system function, being released by and activating receptors on multiple cell types, these transmitters are also associated with many neuropathological conditions, including chronic pain.

Pain is a combination of nociception and perception, and can be influenced by emotional state. Indeed, higher cognitive function plays a role in pain perception and the development of pathological pain. For example, the anterior cingulate cortex (ACC), which is a cortical part of the limbic system, is involved in pain perception (Xu, Wu et al. 2008). In this region, peripheral nerve injury causes increased presynaptic release of glutamate and amplified AMPAR-mediated postsynaptic responses. Furthermore, inhibition of AMPARs alleviates allodynia after injury (Xu, Wu et al. 2008). There is also upregulation of NR2B NMDAR subunit expression in the ACC in chronic pain

models where allodynia can be attenuated by blocking NR2B locally or systemically (Wu, Toyoda et al. 2005). Another supraspinal region involved in chronic pain is the rostral ventral medulla (RVM), which facilitates descending nociceptive pathways (Porreca, Ossipov et al. 2002). In each location, the ACC, the RVM, and the spinal cord, pain perception involves a series of cellular interactions and responses from immune cells, glia and neurons. Signals from glial cells trigger neuronal responses, and vice versa, initiating a complex cascade of cell-cell interactions and feedback mechanisms (Scholz and Woolf 2007).

Acute pain stimuli excite primary nociceptive neurons, which synapse and release glutamate and substance-P onto postsynaptic neurons in the dorsal horn of the spinal cord. Under chronic pain conditions, this synapse exhibits an LTP-like state, known as central sensitization, where increased responses from dorsal horn neurons are elicited by afferent stimulation (Liu and Sandkuhler 1997). This NMDAR-dependent process is correlated with behavioral outputs such as reduced threshold to pain (allodynia) and increased severity of pain sensation (hyperalgesia) (Witting, Kupers et al. 2006). The development of chronic pain is a pathological process that is not fully understood, however experimental models of neuropathic pain (NPP) demonstrate that reactive responses of astrocytes contribute to reduced nociceptive thresholds (Ren and Dubner 2008), a hallmark feature of this condition, but the mechanisms by which this occurs are incompletely defined. Models of NPP also show that microglia and astrocytes residing in the dorsal horn of the spinal cord change from a resting state to an activated state, characterized by hypertrophic morphology, up-regulation of cell-specific proteins, and proliferation (Romero-Sandoval, Chai et al. 2008).

There are two distinct phases in the development of NPP: induction and maintenance. Each of these phases involves distinct cellular responses and can be specifically targeted. Microglia are immunologically active cells thought to mediate the induction of NPP because they quickly become activated by cytokines within the first few days post injury (dpi) (Beggs and Salter 2007). This reactivity occurs in response to ATP accumulation in the dorsal horn of the spinal cord, ipsilateral to the site of nerve injury (Colburn, DeLeo et al. 1997, Coyle 1998). Blocking microglia activity with the inhibitor, minocycline, attenuates pain. Importantly, inhibition of purinergic receptors on microglia alone is able to attenuate pain (Inoue 2008, Inoue 2008, Tozaki-Saitoh, Tsuda et al. 2008). Furthermore, intrathecal application of ATP or ATP-stimulated reactive microglia is sufficient to induce allodynia (Tsuda, Shigemoto-Mogami et al. 2003, Nakagawa, Wakamatsu et al. 2007).

Astrocyte reactivity is delayed compared to microglial reactivity, becoming active at seven dpi, and is thought to be involved in the maintenance phase of NPP, where persistently active astrocytes keep microglia active as well (Zhuang, Gerner et al. 2005, Echeverry, Shi et al. 2008). Delayed administration of fluorocitrate or propentofylline to inhibit glial activity attenuates pain after peripheral nerve injury (Watkins and Maier 2003, Tawfik, Nutile-McMenemy et al. 2007, Wei, Guo et al. 2008). As with microglia, inhibition of purinergic signaling reduces astrocyte reactivity after peripheral nerve injury (Bura et al., 2008), and attenuates pain (Zylka, Sowa et al. 2008). Thus, ATP is a common mediator of pain, in part because it increases glutamate excitation of second order neurons in the spinal cord. Evidence suggests decreased activity of astrocytic

glutamate transporters after nerve injury may also mediate increased excitatory glutamate transmission in the dorsal horn (Sung, Lim et al. 2003, Liaw, Stephens et al. 2005).

ATP release by astrocytes or other cell types may influence microglia (Inoue, Tsuda et al. 2005) or neuronal responses (Nakatsuka and Gu 2001). In turn, neuronal release of glutamate and substance P, both of which increase pain, may stimulate astrocyte release of ATP (Werry, Liu et al. 2006). ATP increases pain by binding to purinergic ligand-gated cationic ion channels (P2XRs) to depolarize dorsal horn neurons. In acute slice preparations of the spinal cord, P2X receptor activation enhanced spontaneous and evoked EPSCs and increased glutamate release in the dorsal horn (Nakatsuka and Gu 2001). Furthermore, selective antagonists of P2XRs attenuated mechanical hypersensitivity after peripheral nerve injury (McGaraughty and Jarvis 2005, Sharp, Reeve et al. 2006, Donnelly-Roberts and Jarvis 2007, McGaraughty, Chu et al. 2007). Under conditions of persistent nociceptive activation, ATP activates P2XRs and slow inactivating GPCR P2YRs on microglia. Although ATP plays an active role in spinal cord signaling, it has a short life span, due to rapid hydrolysis to adenosine.

Adenosine acts on A1Rs in the spinal cord, producing anti-nociceptive effects (Gong, Li et al. 2010). An astrocytic source of adenosine A1R signaling regulates neuronal NMDA receptor (NMDAR) expression and synaptic plasticity in the cortex (Deng, Terunuma et al. 2011). Given that changes in NMDAR expression and activation contribute to the maladaptive synaptic plasticity associated with NPP (Bleakman, Alt et al. 2006), is possible that astrocyte gliotransmission contributes to pain perception. It has been well-established that astrocyte gliotransmission represents a mechanism for fine-tuning synaptic activity (Pascual, Casper et al. 2005, Volterra and Meldolesi 2005,

Panatier, Vallee et al. 2011), but the impact of this process as it relates to physiological behaviors, such as pain sensation, is not well-understood. The development of molecular genetic techniques in which astrocyte function is selectively impaired has made it possible to probe the behavioral impact of these glial cells. For example, using transgenic mice in which SNARE-mediated release of gliotransmitters is selectively attenuated (called "dnSNARE" mice), astrocytes were shown to be critical regulators of A1R-dependent sleep homeostasis (Halassa, Florian et al. 2009).

In Chapter 3, I describe the role of gliotransmission in pain where dnSNARE mice were used to probe the contribution of gliotransmission to physiological and pathological pain sensation. Baseline mechanical nociception was measured as well as nociception after injury. Furthermore, immunohistochemistry was used to quantify reactive astrocytes and microglia in the spinal cords from dnSNARE and wild type mice before and after injury. Understanding the role of astrocytes in nociceptive thresholds may provide future targets for pain management.

**Section 1.7: Cocaine addiction and astrocytes in the striatum**

Drug abuse and addiction are widespread in modern culture, characterized by cravings that result in lack of control over drug seeking and self-administration despite knowledge of the negative effects on quality of living. Cocaine craving extends far beyond the initial stages of withdrawal and increases propensity to relapse (Grimm, Hope et al. 2001). It is thought that the lack of control that leads to compulsive drug taking is because the behavior becomes impervious to the environmental cues that inform the addict of the negative effects of the drug (Goldstein and Volkow 2002, Everitt and Robbins 2005, Kalivas and Volkow 2005, Kalivas 2008). This is attributed to a decrease

in prefrontal cortex (PFC) activation (Goldstein and Volkow 2002) and decreased response to biological rewards (Garavan, Pankiewicz et al. 2000), but increased response to cocaine associated cues (Volkow, Wang et al. 2008) in human subjects addicted to cocaine. Decreased PFC activation results in decreased glutamtergic input to the nucleus accumbens (NAc), and decreased resistance to relapse as well as a strengthened, independently operating striatal activation, associated with habitual drug seeking behavior. Rodent models show addictive-like behaviors involving increased energy expenditure towards acquiring cocaine, indicative of a strong desire to obtain the reward despite adverse consequences (Roberts, Morgan et al. 2007). Models of addiction in rodents have allowed us to study and understand many physiological mechanisms tied to cocaine related behaviors in humans.

The reward circuitry is composed of the ventral tegmental area (VTA), which contains dopaminergic neurons that project to the NAc and PFC. PFC neurons are glutamatergic, and innervate GABAergic medium spiny neurons (MSNs) which, in turn, project back to the VTA. MSNs in the NAc also project to the dorsal striatum to influence movement. Two mostly non-overlapping groups of cells project to different parts of the dorsal striatum. The direct pathway contains MSNs expressing dopamine D1 receptors (D1Rs) and initiates movement. The indirect pathway contains MSNs expressing D2Rs and inhibits movement. Neuroimaging studies in human addicts show decreased D2R levels in the striatum (Volkow, Fowler et al. 2004). Decreased activation of the indirect pathway to the dorsal striatum may explain the impulsivity phenotype in addicted patients.

Immediate cocaine exposure inhibits reuptake of dopamine and prolongs its lifetime in the synaptic cleft. Dopamine is a neuromodulator that can enhance or dampen the effect of neurotransmission, depending on the synapse at which it is acting. In the mesolimbic dopamine system, dopamine serves to reinforce behaviors that evoke its release (Baik 2013). A single exposure of cocaine induces LTP in VTA dopamine neurons (Ungless et al., 2001). Moreover, persistent cocaine exposure, and influence of VTA neurons, is required to induce physiological changes in NAc neurons (Mameli, Halbout et al. 2009). LTD and LTP are attenuated in the core of the NAc when PFC fibers are stimulated after withdrawal from cocaine exposure (Martin, Chen et al. 2006, Kauer and Malenka 2007, Kourrich, Rothwell et al. 2007, Kasanetz, Deroche-Gamonet et al. 2010). These changes in plasticity are predictive of high relapse rates in mice. The following paragraphs will review the underlying mechanisms by which cocaine mediates relapse related plasticity in the NAc.

The direct effect of cocaine is increased dopamine in the VTA. Dopamine stimulation of D1 MSNs increases AMPAR membrane insertion and function (Price, Kim et al. 1999, Snyder, Allen et al. 2000, Sun, Zhao et al. 2005). D1R insertion of AMPARs specifically mediates relapse to cocaine self-administration (Pascoli, Terrier et al. 2014). Stimulation of D1Rs activates signaling cascades leading to increased intracellular cAMP production and subsequent degradation to adenosine (Hack and Christie 2003). Adenosine signaling modulates many cocaine-induced changes in cellular physiology and behavior (O'Neill, Hobson et al. 2014). For example, adenosine activity in the NAc decreases locomotor sensitization at the point of cocaine challenge (Hobson, Merritt et al. 2012). Chronic cocaine increases adenosine tone in the NAc (Fiorillo and Williams

2000), but pre-synaptic adenosine receptors in the NAc have decreased sensitivity (Manzoni, Pujalte et al. 1998) and receptor surface expression is reduced (Toda, Alguacil et al. 2003). Adenosine A1Rs dimerize with D1Rs and inhibit D1R signaling cascades when activated (Azdad, Gall et al. 2009). A1R stimulation (likely post synaptically on MSNs) directly opposes cocaine induced D1R activation to increase AMPAR phosphorylation and expression in the NAc and dampens cocaine-seeking behavior (Hobson, O'Neill et al. 2013).

Another type of adenosine receptor, the A2AR is sensitive to higher levels of adenosine than A1Rs. Therefore under conditions of increased adenosine tone, such as cocaine withdrawal, selective activation of A2ARs may occur (Ferre, Diamond et al. 2007). While A1R activation is mainly inhibitory, A2ARs, which are densely expressed in the striatum, enhance presynaptic glutamate release, thereby increasing the output of D2R expressing MSNs (Ciruela, Casado et al. 2006, Schiffmann, Fisone et al. 2007). But A2ARs also form inhibitory dimers with D2Rs postsynaptically (Ferre, Quiroz et al. 2008). Inactivation of A2ARs under control (no cocaine) conditions attenuates LTP in the NAc (d'Alcantara, Ledent et al. 2001). Withdrawal from chronic cocaine results in a decrease in A2ARs (Marcellino, Roberts et al. 2007), suggesting that a lack of A2AR activation may be linked to the altered plasticity in the NAc that mediates relapse behavior. Indeed, A2AR activation inhibits cocaine self-administration (Knapp, Foye et al. 2001) and A2AR antagonists reinstate cocaine self-administration (Weerts and Griffiths 2003). It is important to note that one study found the outcome of A2AR inhibition to be brain region specific. When A2ARs were knocked out in the striatum,

cocaine induced locomotor behavior was enhanced, but knocking out A2ARs in the forebrain attenuated locomotor activity induced by cocaine (Shen, Coelho et al. 2008).

On postsynaptic D2R MSNs, A2ARs form synergistic dimers with mGluR5s (Ferre, Karcz-Kubicha et al. 2002). Type I mGluRs (mGluR1/5) are down regulated after withdrawal from chronic cocaine treatment (Mitrano, Arnold et al. 2008). Furthermore, mGluR5 has been linked to LTP in the NAc (Schotanus and Chergui 2008), and mGluR1 dependent LTD can reverse cocaine-induced redistribution of AMPARs (Bellone and Luscher 2006), suggesting a role for type I mGluRs in mediating cocaine-induced changes in plasticity as well. Indeed, mGluR5 antagonists decrease self-administration (Kenny, Boutrel et al. 2005) and reinstatement of cocaine seeking (Backstrom and Hyytia 2006, Kumaresan, Yuan et al. 2009) and inhibition of mGluR5 dose-dependently decreased CPP for cocaine (McGeehan and Olive 2003). NAc astrocytes elevate their intracellular $Ca^{2+}$ in response to mGluR5 activation (D'Ascenzo, Fellin et al. 2007), suggesting astrocytes may be involved in mGluR5-dependent changes after cocaine exposure. Indeed, after withdrawal from chronic cocaine exposure, astrocytes in the NAc become reactive, shown by up-regulation of GFAP, a response to mGluR5 activation (Bowers and Kalivas 2003).

Withdrawal from chronic cocaine exposure causes a 50% reduction in extracellular glutamate in the NAc (Kalivas, McFarland et al. 2003, Kalivas 2009). This is a predictor of addictive behavior since deregulation of glutamate signaling exists only in mice that exhibit drug-seeking behaviors (Pierce, Bell et al. 1996, McFarland, Lapish et al. 2003, McFarland, Davidge et al. 2004). After prolonged withdrawal and re-exposure to cocaine, extra-synaptic glutamate increases (Baker, McFarland et al. 2003,

Miguens, Del Olmo et al. 2008). Specifically, microdialysis studies show that PFC-released glutamate is increased in the NAc after drug reinstatement (McFarland, Lapish et al. 2003, Madayag, Lobner et al. 2007). The changes in extracellular glutamate seen after withdrawal from chronic cocaine are likely due to a deregulation of the release of glutamate from PFC afferents, inhibition of the astrocytic cysteine-glutamate exchanger (Kalivas 2009), decreased activity of the astrocytic glutamate transporter, GLT-1 activity and expression (Sari, Smith et al. 2009, Knackstedt, Melendez et al. 2010), and possibly other mechanisms involving astrocytes.

The effects of cocaine on glutamate in the NAc are two-fold. Both synaptic and extra-synaptic glutamate signaling are altered. Group II mGluRs inhibit presynaptic glutamate release and are downregulated after chronic cocaine treatment (Xi, Ramamoorthy et al. 2002). As a consequence of decreased expression and lowered extrasynaptic glutamate acting on presynaptic group II mGluRs, synaptic release of glutamate is increased after withdrawal from chronic cocaine exposure (Kalivas 2009). Restoration of group II mGluR function with an mGluR2/3 agonist attenuates cocaine self-administration (Adewale, Platt et al. 2006) and reinstatement of drug seeking (Baptista, Martin-Fardon et al. 2004), suggesting increased synaptic glutamate release is involved in relapse behavior. These cocaine-induced changes in synaptic glutamate initiate homeostatic synaptic scaling in NAc medium spiny neurons (MSNs) that lead to impairments in plasticity (Huang, Yeh et al. 2011).

Cocaine-induced changes in glutamate levels are associated with compound adaptive changes in MSNs. Early withdrawal (1-3 days) from chronic cocaine administration results in spinogenesis, upregulation of NMDARs, and increased

expression of silent synapses (Huang, Lin et al. 2009). At the same withdrawal time point, the intrinsic membrane excitability of MSNs is decreased, specifically decreasing action potential output after glutamate stimulation (White, Hu et al. 1995, Keys, Mark et al. 1998, Baker, McFarland et al. 2003, Martin, Chen et al. 2006, Schramm-Sapyta, Olsen et al. 2006, Moussawi, Pacchioni et al. 2009, Kasanetz, Deroche-Gamonet et al. 2010). MSNs are not intrinsically active and rely heavily on excitatory input to elicit action potentials and are thus at the mercy of changes in glutamate (Sesack and Grace 2010). The current understanding of the synaptic changes that occur after withdrawal from cocaine are largely thought to be a result of AMPAR trafficking in response to the altered pre-synaptic release of glutamate. Withdrawal from chronic cocaine causes an increase in surface expression of AMPARs to adapt to decreased glutamatergic signaling. Conversely, the reinstatement of cocaine restores glutamatergic signaling, which results in internalization of AMPAR after 24 hours (Boudreau, Reimers et al. 2007).

A major consequence of extended withdrawal from cocaine is increased AMPAR expression and function (Malinow and Malenka 2002, Boudreau and Wolf 2005). These changes in AMPAR trafficking are thought to underlie the impaired plasticity seen in the NAc when PFC fibers are stimulated in slices taken after withdrawal from cocaine exposure (Martin, Chen et al. 2006, Kourrich, Rothwell et al. 2007, Kasanetz, Deroche-Gamonet et al. 2010). It is hypothesized that the trafficking of AMPAR to the post-synaptic density during withdrawal potentiates the synapse and occludes LTP while it attenuates LTD (Martin, Chen et al. 2006, Moussawi, Pacchioni et al. 2009). There is substantial evidence that PFC activation is required for reinstatement of cocaine seeking behavior (Capriles, Rodaros et al. 2003, Sun and Rebec 2005). For example, AMPAR

agonists reinstate cocaine seeking (Cornish, Duffy et al. 1999, Cornish and Kalivas 2000, Suto, Tanabe et al. 2004, Ping, Xi et al. 2008), while inhibiting AMPA in the NAc impairs cue- and cocaine-induced reinstatement (Park, Bari et al. 2002, Backstrom and Hyytia 2007, Conrad, Tseng et al. 2008). Specifically, cocaine relapse requires PFC activation of AMPARs in the NAc, as inactivation of either of these regions attenuates relapse behavior (Cornish, Duffy et al. 1999, Cornish and Kalivas 2000, Di Ciano and Everitt 2001, Park, Bari et al. 2002, McFarland, Lapish et al. 2003, Backstrom and Hyytia 2007, Bachtell and Self 2008). Furthermore, cocaine reinstatement restores AMPAR-mediated glutamate transmission in the NAc to pre-withdrawal conditions (Park, Bari et al. 2002). Reinstatement after withdrawal or extinction from chronic cocaine results in a decrease in the synaptic AMPAR/NMDAR ratio (Thomas, Beurrier et al. 2001, Boudreau and Wolf 2005, Boudreau, Reimers et al. 2007, Kourrich, Rothwell et al. 2007, Conrad, Tseng et al. 2008, Schumann and Yaka 2009). However, reinstatement of cocaine after withdrawal only reverses AMPAR levels to pre-cocaine levels for a limited time if cocaine administration does not persist (Ferrario, Li et al. 2010, Wolf and Ferrario 2010). It is thought that one mechanism by which cocaine reinstatement decreases AMPAR expression is by acutely increasing glutamate levels in the NAc (Boudreau, Reimers et al. 2007, Kourrich, Rothwell et al. 2007). In agreement with this hypothesis, in cultured NAc neurons AMPARs were internalized after glutamate levels were increased (Mangiavacchi and Wolf 2004). Furthermore, in cocaine-withdrawn mice, AMPAR internalization requires AMPAR stimulation, as blockade of AMPARs with CNQX during cocaine reinstatement blocked AMPAR internalization (Bachtell and Self 2008). Just as plasticity in the hippocampus acts via NMDAR activation to govern

AMPAR trafficking, there is evidence for cocaine-induced plasticity using this mechanism in the NAc (Schumann and Yaka 2009). Although the changes in glutamatergic signaling in the NAc after exposure to cocaine are well documented, the mechanisms by which these changes occur are poorly understood.

In the cortex, NMDAR expression levels are reduced in a mouse model of attenuated gliotransmission (Deng, Terunuma et al. 2011). In addition to regulating NMDAR expression via postsynaptic A1R activation on neurons, astrocytic adenosine regulates evoked glutamate release by tonic inhibition of presynaptic A1Rs in the hippocampus (Schmitt, Sims et al. 2012). Notably, pre-synaptic activation of A1Rs on PFC terminals dramatically decreases glutamate and dopamine release in the NAc (Uchimura and North 1991, Wu and Saggau 1997, Hartse 2011), but whether this source of adenosine is astrocytic is unknown.

In a behavioral model of relapse to cocaine seeking, mice with attenuated gliotransmission fail to exhibit cue-induced relapse behavior (Turner, Ecke et al. 2013). Given that gliotransmission is necessary for expression of relapse behavior and that A1R activation and NMDAR mediated plasticity in the NAc is altered in models of cocaine exposure, it is important to understand the effects of attenuated gliotransmission on the physiological function of these receptors under baseline conditions prior to cocaine exposure.

In chapter 4, I describe the investigation of gliotransmission in basal NAc physiology. To determine the extent to which astrocyte gliotransmission contributes to tonic activation of A1Rs in the NAc, adenosine tone was measured in wild type and

dnSNARE mice. NAc physiology was further investigated by measuring MSN excitability in wild type and dnSNARE mice. Finally, the contribution of gliotransmission to glutamate receptor function was measured by recording mEPSCs and evoked AMPA/NMDA currents.

**Section 1.8: Sleep and Astrocyte Function**

Sleep is a vital function in all mammals. It is critical for proper brain function, including memory formation. Although we do not know the exact role of sleep in brain function, we are painfully aware of the consequences of sleep deprivation as well as the correlations between disrupted sleep and neurodegenerative and psychiatric disorders. Sleep is a reversible state of decreased arousal and is characterized by specific stages, such as rapid eye movement (REM) and non-REM (NREM). Each vigilance state is characterized by specific polysomnographic measurements obtained from electroencephalogram (EEG), to measure cortical neuronal activity, electromyogram (EMG), to measure muscle tone, and electro-oculogam (EOG), to measure eye movements. Wakefulness is characterized by low power desynchronized EEG activity, high EMG activity, and controlled eye movements. When the transition from wake to sleep occurs, NREM is the first stage and is characterized by higher amplitude, slow synchronized EEG activity and low EMG power with few involuntary spikes in activity. Finally, once the transition from NREM to REM is made, EEG activity is characterized by low amplitude, fast activity. EMG power is almost non-existent due to inhibition of muscle tone, and the EOG measures quick, involuntary rapid eye movements. The following section will explain the three main vigilance states and how they are regulated.

In order to distinguish sleep from wake, we must review the physiology of wakefulness, the state of active acquisition of information from the environment. Wake is characterized by activity across all frequency ranges. The prominent frequencies in the EEG during wake are low amplitude, high frequency beta (15-30 Hz) and gamma (30-120 Hz) waves. Many cortical oscillations rely on sub-cortical input as a driving force. Gamma waves are unique in that they are synchronized by interneurons within the cortex that clamp cortical pyramidal neurons to oscillate at this high frequency (Csicsvari, Jamieson et al. 2003). However, gamma does rely on sub-cortical input as well (Urbano, Kezunovic et al. 2012, Urbano, D'Onofrio et al. 2014). It is thought that different sub-regions of the cortex are discretely controlled and attention directed tasks may involve switching between regions (Whittington, Cunningham et al. 2011). There are many brain regions that act to promote the physiology correlated with a waking state of the cortex. The brain stem contains monoaminergic neurons in the locus coeruleus (norepinepherine) and the raphe nuclei (serotonin), the hypothalamus contains histaminergic nuclei and orexin neurons which are inhibited by A1R activation, and the brainstem and basal forebrain (BF) contain cholinergic neurons (Stenberg 2007, Brown, Basheer et al. 2012). The BF cholinergic system promotes wake, inhibits sleep active brain regions, and is critical for cognitive function and active brain physiology. Indeed, cholinergic activation can induce gamma oscillations in brain slices (Fisahn, Pike et al. 1998) and alpha rhythms (8-14 Hz) in thalamic slices (Lorincz, Crunelli et al. 2008). Alpha rhythms are also present during wake and are generated by thalamocortical neurons (Hughes and Crunelli 2005). Less is known about beta oscillations, but evidence suggests they also play a role in synchronizing cortical activity (Kopell, Ermentrout et al. 2000). Theta

frequency (5-8 Hz) waves are involved in directed movement and memory formation (Buzsaki and Draguhn 2004) and are prominently represented in the hippocampus (Buzsaki 2002) and driven by the medial septum, which receives input from the supramammillary nucleus and from the nucleus pontine oralis (Brown, Basheer et al. 2012). Theta oscillations are also a prominent feature during REM sleep and will be discussed in more detail in that context.

NREM sleep is broken into four stages, representing progression of depth, in humans, but is measured as a single state in rodents. Briefly, in humans, stage one sleep represents light sleep and EEG patterns are similar to wake. During stages two and 3, sleep spindles (thalamocortical) and K-complexes (a single slow wave oscillation) are present until full progression into slow wave activity occurs in stage 4 of NREM sleep (Brown, Basheer et al. 2012). In mice, Delta waves are driven by corticothalamic to thalamocortical projection loops that oscillate (de Andres, Garzon et al. 2011). NREM sleep is dominated by high amplitude, slow wave delta oscillations (0.5-4 Hz). Sleep spindles are also detectable from mouse brain recordings (Vyazovskiy, Achermann et al. 2004) but K-complexes are not reported. NREM is driven by inactivation of the wake-promoting cholinergic basal forebrain by adenosine acting on post-synaptic A1Rs and inhibition of release by activation of pre-synaptic A1Rs (Rainnie, Grunze et al. 1994, Thakkar, Delgiacco et al. 2003, Basheer, Strecker et al. 2004, Hawryluk, Ferrari et al. 2012, Yang, Franciosi et al. 2013). The ventrolateral preoptic nucleus (VLPO), a major sleep-promoting brain region, is also disinhibited presynaptically and activated by A2ARs postsynaptically (Gallopin, Luppi et al. 2005). The depth of NREM, measured by EEG amplitude (power) in the delta frequency, is heavily regulated by sleep homeostasis,

which will be discussed in a later section. NREM sleep depth is also affected by stress and infection (Mullington, Korth et al. 2000, Majde and Krueger 2005). SNARE-dependent gliotransmission is required for the increase in NREM sleep power induced by injection of lipopolysaccharide to model inflammatory infection (Nadjar, Blutstein et al. 2013).

Humans and mice normally follow a transition from NREM to REM sleep. Direct wake to REM transitions are only seen in narcoleptic patients and other conditions of decreased orexin signaling. As seen by the EEG, REM is somewhat similar to wake, with low amplitude, fast EEG oscillations in the 5-8 Hz (theta) range (Platt and Riedel 2011). REM is generated when cholinergic neurons in the pons become dis-inhibited (Colgin 2013). REM and wake active cholinergic neurons project to the thalamus (Lu and Zee 2010) and basal forebrain to the hippocampus (Everitt and Robbins 1997). Thus, cholinergic activation of pons neurons drives prominent theta rhythms in the hippocampus (McCarley and Massaquoi 1992, Buzsàki 2002, McCarley 2007, Pignatelli, Beyeler et al. 2012, Carroll and Landau 2014). Furthermore, activation of the pons with cholinergic agonists increases time spent in REM (Bezzi, Carmignoto et al. 1998).

Norepinephrine, serotonin and acetylcholine are released by neurons in the pons (Jones 1991). The reciprocal interaction model (McCarley and Massaquoi 1992) states that the two groups of neurons in the pons, the REM active (cholinergic) and REM inactive (norepinepherine and serotonin) neurons, are self-propagating and one group inhibits the other, so that once the switch to REM begins to flip, it does so completely and without a transition state. The REM state is thought to terminate as REM-ON neurons allow for slow depolarizations in REM-OFF neurons, which promote transitions

back to NREM once they begin to fire. What causes the switch from one state to the next is not completely understood.

The circadian regulation of sleep and wake relies on external cues such as sunlight that provides information to sleep centers through the supra-chiasmatic nucleus of the thalamus, as well as oscillations that are internally generated. Astrocytes play a role in internally generated circadian rhythms (Suh and Jackson 2007). Adenosine is a potent somnogen that is metabolized more quickly at night due to upregulation of enzymes like adenosine kinase, 5'-nucleotidase, and adenosine deaminase (Chagoya de Sanchez, Hernandez Munoz et al. 1993). However, none of the enzymes involved in adenosine metabolism have been found to fluctuate after periods of sleep deprivation, suggesting degradation of adenosine is time of day dependent, but not homeostatically regulated (Porkka-Heiskanen and Kalinchuk 2011). The accumulation of adenosine and adenosine receptor expression however are homeostatically regulated. These findings have been reproduced in many studies probing the sleep homeostat by sleep depriving mice.

Sleep deprivation is correlated with moderate to severe memory impairments, decreased immune function, and increased risk of cardiac disease, obesity, hallucinations and even seizures. Sleep homeostasis is in place to ensure sleep debt is paid off to avoid the consequences of sleep loss. Sleep homeostasis may ensure synaptic homeostasis in the brain (Tononi and Cirelli 2003, Tononi and Cirelli 2006), maintaining a delicate balance of synaptic strength. The homeostatic drive to sleep results in an increase in NREM and REM sleep after periods of sleep loss. Sleep duration is highly correlated with the duration of wakefulness prior to sleep (Kalinchuk, McCarley et al. 2011).

Additionally, prolonged wake results in increased delta power, or sleep pressure, that predicts sleep intensity. Moreover, adenosine accumulation is tied to these increases in sleep pressure and sleep duration after prolonged wake. During recovery sleep after sleep deprivation, adenosine tone and delta power slowly return to baseline levels (Blutstein and Haydon 2013). Collectively, the rise and fall of adenosine and sleep pressure with wake and subsequent sleep is how sleep homeostasis is maintained. An acute (3-6hr) or prolonged (12-24hr) period of sleep deprivation (SD) beginning at the onset of the inactive phase is the gold standard for probing sleep homeostasis in mice. Acute SD robustly increases rebound sleep and the sleep pressure shown by increased amplitude of NREM delta power during recovery sleep. Many molecules increase in the brain with wakefulness, and many are correlated with the increased activity and energy metabolism associated with increased wake. For example, nitric oxide, cytokines, BDNF, norepinephrine, and adenosine are all increased with prolonged wake and are all thought to play a role in sleep homeostasis (Porkka-Heiskanen 2013). Adenosine is the most widely studied somnogen involved in the homeostatic drive to sleep and will be the focus of this section. A recent opinion in neurobiology is that astrocytes are well positioned to detect increases in many wake-active substances such as BDNF and norepinephrine with an increase in intracellular $Ca^{2+}$, and subsequent release of ATP, which is degraded to adenosine (Frank 2013).

Activation of the A1R is critical for the homeostatic action of adenosine as a conditional A1R knockout mouse failed to exhibit increased delta power after 4hr SD (Bjorness, Kelly et al. 2009). Adenosine activation in the BF is of particular importance, because this is where A1R activation inhibits wake active neurons. One study revealed

that knocking down the A1R in the BF significantly attenuated recovery sleep after SD (Thakkar, Winston et al. 2003). Adenosine is cleared from the extracellular space by equilibrative nucleoside transporters (ENTs). When ENT1 was blocked in the BF, total sleep, including NREM and REM was increased (Porkka-Heiskanen, Strecker et al. 1997). Other ways of increasing adenosine are through targeting its metabolic processing. Inhibitors of adenosine deaminase, to prevent breakdown to inosine, (Radulovacki, Virus et al. 1983), and adenosine kinase, to prevent phosphorylation to adenosine monophosphate (Palchykova, Winsky-Sommerer et al. 2010), increase wake and decrease sleep. Furthermore, ectonucleotidases break down ATP to AMP to adenosine in the extracellular space and ectonucleotidase CD73 knock-out mice do not exhibit sleep rebound after SD (Zielinski, Taishi et al. 2012). Interestingly, humans that have adenosine deaminase mutations have increased NREM sleep (Retey, Adam et al. 2005), presumably due to increased adenosine levels. Also in the BF, prolonged (12-24hr) SD increases A1R mRNA, perhaps due to increased adenosine levels throughout SD (Basheer, Strecker et al. 2004). Later studies showed that A1R density, measured with a binding assay, is also increased after 24hr SD (Basheer, Bauer et al. 2007, Elmenhorst, Basheer et al. 2009). These results are supported by increased A1R binding in PET imaging studies performed on sleep-deprived humans (Elmenhorst, Meyer et al. 2007).

Adenosine accumulation undoubtedly increases with prolonged wakefulness and promotes sleep through inhibitory action in the BF, where A1R expression dominates. Still, other brain regions are involved in mediating the somnogenic effects of A1R stimulation, and in some regions A2ARs allow for excitation of sleep promoting neurons (Bjorness and Greene 2009). The VLPO is a sleep promoting brain region that becomes

more active after application of adenosine. This is due to an inhibitory action on presynaptic GABA input to these cells (Chamberlin, Arrigoni et al. 2003). Additionally, VLPO neurons express excitatory A2ARs and are indeed activated by adenosine (Gallopin, Luppi et al. 2005). Adenosine inhibits wake-promoting release of histamine from the hypothalamus to the cortex via A1R activation (Oishi, Huang et al. 2008). Again in the hypothalamus, A1R activation inhibits wake-promoting hypocretin neurons and A1R antagonist microinjection delayed NREM onset (Thakkar, Engemann et al. 2008).

Recent work has also shown wakefulness-dependent increases in adenosine in the somatosensory cortex. The source of this adenosine accumulation was found to depend on SNARE-mediated release of ATP from astrocytes (Blutstein and Haydon 2013), but it may be due to AMP accumulation as well (Rittiner, Korboukh et al. 2012) as adenosine tone was measured via A1R activation. Furthermore, the deficit in adenosine in mice with attenuated gliotransmission resulted in decreased sleep pressure accumulation after SD as well as decreased recovery sleep, measured by total sleep time and NREM bout duration (Halassa, Florian et al. 2009). This was attributed to decreased A1R activation as A1R antagonism mimicked the effect of attenuated gliotransmisison and attenuated sleep pressure accumulation. Additionally, a later study measured A1R adenosine tone in the hippocampus after sleep deprivation and found that mice with attenuated gliotransmission did not exhibit a rise in adenosine tone, where wild type mice accumulated adenosine tone over a six hour SD period. This conclusion was made based on data obtained from *in situ* field recordings in the hippocampus as well as *in vivo* local field potentials in the cortex. Furthermore, *in situ* biosensors were used as a more direct measurement of adenosine and confirmed that SD caused increased adenosine in extracted hippocampi

from wild type mice, but not mice with attenuated gliotransmission (Schmitt, Sims et al. 2012).

NREM sleep has largely been tied to increases in adenosine, but adenosine signaling also influences REM sleep. Injection of an A1R agonist into the pontine reticular formation decreased wake and increased REM sleep (Marks and Birabil 1998). Furthermore, A2ARs also play a role in REM sleep generation as cholinergic neurons in the pons increase their firing in response to A2AR agonists, resulting in increased REM (and NREM) sleep (Coleman, Baghdoyan et al. 2006). Theta power is also increased after sleep deprivation and is correlated with sleep drive (Finelli, Baumann et al. 2000, Vyazovskiy and Tobler 2005). NREM and REM sleep are both increased after recovery from prolonged wakefulness (Vyazovskiy and Delogu 2014).

Astrocytes have been shown to influence brain activity by means of neuromodulation. Astrocytes affect plasticity and cortical oscillations as well as sleep homeostasis (Fellin, Halassa et al. 2009, Halassa, Florian et al. 2009, Lee, Ghetti et al. 2014). Using genetically modified mice that have attenuated gliotransmission, the dnSNARE mouse, it was shown that the gliotransmitter adenosine (derived from ATP) was critical for the accumulation of sleep pressure, otherwise known as slow wave delta power (Halassa, Florian et al. 2009). Considering that calcium signaling is required for neurotransmission, and there is extensive evidence that it is required for gliotransmission (Porter and McCarthy 1997, Araque, Sanzgiri et al. 1998, Buzsàki 2002, Hua, Malarkey et al. 2004, Kreft, Stenovec et al. 2004, Chen, Wang et al. 2005, Pryazhnikov and Khiroug 2008, Paukert, Agarwal et al. 2014), we asked whether we could replicate the dnSNARE phenotype of impaired sleep homeostasis in a mouse model of disrupted

astrocytic $IP_3/Ca^{2+}$ signaling. We overexpressed a Venus tagged IP3 5'phosphatase (VIPP) transgene selectively in astrocytes to enhance the metabolism of $IP_3$ to $IP_2$ in order to attenuate $IP_3$-mediated $Ca^{2+}$ release in this cell type. We measured NREM And REM sleep time as well as delta and theta power to assess sleep intensity. The results of this study suggest a previously unrecognized and unique role for astrocytic $IP_3/Ca^{2+}$ signaling in neuromodulation, independent of SNARE-mediated release from astrocytes. Moreover, these results demonstrate the importance of the astrocyte, a glial cell sub-type, in the control of the generation of sleep states and brain rhythms.

## Chapter 2: Materials and Methods

dnSNARE mouse:

The tTA tet-O system was used to create inducible astrocyte-specific expression of dnSNARE, a dominant negative mutation of the synaptobrevin II protein in the SNARE complex. Two mouse lines were crossed to achieve inducible expression of dnSNARE selectively in astrocytes, the tetracycline transactivator line was driven by the astrocyte specific GFAP promoter, and the dnSNARE, GFP and Lac-Z reporter genes were driven by the tetracycline operator (Fellin, Pascual et al. 2004, Pascual, Casper et al. 2005, Halassa, Florian et al. 2009). Mice were raised on doxycycline to prevent transgene expression during development and thus to prevent potential adaptations to transgene expression. Doxycycline was removed from the diet at weaning to permit transgene expression in the mature animal. Two weeks post doxycycline removal, abundant transgene expression was evident throughout the brain and spinal cord. Male animals were tested at 8-10 weeks of age and were housed on a 12hr/12hr light/dark cycle. All procedures were in strict accordance with National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Tufts University Institutional Animal Care and Use Committee.

Behavioral Testing:

Von Frey monofilaments were used to test baseline mechanical nociception as well as allodynia after SNI. The monofilaments were used to apply controlled force to the lateral portion of the left hind limb, similar to what has been previously described (Decosterd and Woolf 2000, Bourquin, Suveges et al. 2006, Scholz, Abele et al. 2008). Animals were tested in wire mesh chambers to which they were habituated 5 days before testing.

Pain threshold was recorded when animals elicited 4-5/10 pain responses (defined by rapid paw withdrawals often with paw shaking or licking) for a given monofilament ranging from 0.02g to 2g. A subset of animals were tested for NPP after SNI. Surgery was performed under isoflurane anesthesia and the tibial and peroneal branches of the sciatic nerve were ligated while the sural branch was spared (Bourquin, Suveges et al. 2006). Beginning 3dpi, changes in mechanical nociceptive responses were measured using von Frey's fiber test. Measurements were taken as described above, and trials were repeated on 3, 7, 10, 14, 21, and 28dpi. Criteria for exclusion identified outliers (>2 standard deviations from within group mean), which were excluded from this study (2 dnSNARE, 1 WT). Upon completion of the experiments the animals were euthanized by isoflurane followed by cardiac perfusion, for histology.

Immunohistochemistry:

Pain study: Mice (3 wild-type and 3 dnSNARE) were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) and post-fixed for 24 hours. Spinal cords were removed from spinal column and placed in 10% and then 30% sucrose. The lumbar spinal cord (L4-L6) was sectioned on a sliding microtome at a thickness of 40mm and placed in PBS. Sections were stained with Rabbit anti-Iba-1 (Wako, 1:1000) chicken anti-GFAP (abcam, 1:1000), and mouse anti-Neu-N (chemicon, 1:1000). Secondary antibodies conjugated to Alexafluor were used. Goat anti-rabbit Alexa633, goat anti-chicken Alexa546 and goat anti-mouse Alexa633 were used, all at a 1:500 dilution. At least three representative sections were taken from each condition for quantification.

Sleep study: Mice (3 wild-type, 3 VIPP) were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) and post-fixed for 24h. Brains were

cryoprotected in 30% sucrose. Fixed, frozen sections (40μm) were cut using a cryostat (Microm HM 525, Thermo Scientific). Sections were stained for Beta-galactosidase expression using X-Gal staining solution (Millipore). Images of the sections were collected using a Nikon E800 bright field microscope. RGB histograms were linearly adjusted for all images collected using Adobe Photoshop. Astrocyte-specific staining was determined by staining sections with chicken anti-GFAP (Abcam, 1:1000), mouse anti-Neu-N (chemicon, 1:1000), and rabbit anti-Beta galactosidase (Molecular Probes). Secondary antibodies conjugated to Alexa fluor dyes were used. Goat anti-mouse Alexa488, goat anti-rabbit Alexa546, and goat anti-chicken Alexa633 were used, all at 1:1000 dilutions. Three representative sections were taken from each condition for quantification of cell-type specific expression. DAPI hard set Vectashield mounting medium (Vector Laboratories) was used.

Confocal imaging:

Confocal images were acquired using a Nikon Eclipse Ti (or Ti-E) microscope. The substantia gelatinosa of the dorsal horn was imaged in the pain study. For imaging of reactive changes in astrocytes and microglia in the pain study, maximum intensity projections were created from 10μm Z-stacks taken with a 20x objective (0.75NA). ImageJ software was used to measure the percent area above threshold for each antibody. Background values were subtracted. For counting of co-localization, single plane optical section images (60x objective, 1.40NA) were taken and each clearly defined cell was counted as EGFP positive or negative based on localization of astrocyte, microglia or neuron markers with DAPI labeled nuclei. For counting of co-localization for Beta-galactosidase colocalization in the sleep study, cells were identified as positive or

negative based on localization of astrocyte or neuron markers with DAPI labeled nuclei. Images were linearly adjusted for brightness and contrast.

VIPP mouse:

The VIPP mouse line was created by fusing the inositol-1,4,5-trisphosphate 5-phosphatase construct (F, L et al. 1997, Evanko and Haydon 2005) to the Venus construct (Evanko and Haydon 2005). Tet-O VIPP mice were crossed with GFAP tTA mice to ensure astrocyte specific expression of VIPP. Transgene expression was confirmed by Venus reporter fluorescence or Beta-galactosidase expression. All animals were housed on a 12h light/dark cycle and bred with 40 mg/kg doxycycline (Dox, Bioserv, Frenchtown, NJ) in the diet, and maintained on Dox until weaning to suppress transgene expression during development. Experimental mice were aged 8-12 weeks and were given standard chow and water *ad libitum*. Control experiments were performed on mice lacking one or more transgenes. All experiments were performed with the approval of the Tufts University Institutional Animal Care and Use Committee, and under the guidelines defined by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Electroencephalogram/electromyogram implantation surgery and data collection:

Mice were anesthetized with isoflurane and placed into a stereotaxic frame. For implantation of electroencephalogram (EEG) electrodes, the skull surface was exposed and four insulated wire electrodes were placed and screwed as previously described (Clasadonte, McIver et al. 2014). After surgery, mice were intraperitoneally injected with buprenorphine (0.08 mg/kg) and lactated Ringer's solution, and fed moistened rodent

food. After 5d of postoperative recovery, lightweight recording cables were connected to the head implants and mice were placed in Circular Plexiglas cages (Pinnacle) containing water and food *ad libitum* and acclimated for a week. Following acclimation, EEG/EMG activity was continuously monitored for 48h. Baseline EEG/EMG activity starting at ZT0 was measured for 24h (baseline), followed by 6h of sleep deprivation enforced by gentle handling and perturbation of bedding. EEG/EMG activity was monitored during the 6h of sleep deprivation and 18h of recovery sleep. During data acquisition, EEG signals were high pass filtered at 0.5Hz and low pass filtered at 40Hz. EMG signals were high pass filtered at 0.5Hz and low pass filtered at 100Hz. The amplifier system (Pinnacle Technology, Inc.), sampled at 250Hz with a PAL 8400 data acquisition system (Pinnacle). Data was stored on a personal computer.

Vigilance state scoring and analysis:

Sleep stages were scored visually based on 4s epochs by a trained experimenter using SleepSign for Animal software (Kissei Comtec). Wakefulness consisted of low-amplitude, high-frequency EEG and high EMG activity; rapid eye movement (REM) sleep consisted of low-amplitude, desynchronized EEG with low EMG activity; and Non REM (NREM) sleep consisted of high-amplitude, low-frequency EEG with little EMG modulation. Brief awakenings defined as uninterrupted waking episodes of 1–9 epochs were not included in the analysis. Epochs containing movement artifacts were included in the state totals, but excluded from subsequent spectral analysis. After assignments of state scores, the amount of each state (expressed as a percentage of the total recording time in 1hr time bins) and their duration were measured. EEG power spectra of consecutive 4s

epochs (fast Fourier transform routine; Hanning window) were calculated. Power spectra were normalized to data in the 0.5 to 1.5Hz frequency range because these values were not different between genotypes. The EEG power during NREM sleep from 0.5 to 1.5Hz was defined as low frequency slow wave activity (lf-SWA) and was used as a quantitative measure of sleep pressure and homeostatic sleep drive. The EEG power of SWA during NREM sleep was used to assess hour by hour sleep power. Hour by hour lf-SWA was normalized to the last four hours of the baseline day. The EEG power during REM sleep from 5.0 to 8.0Hz was defined as theta activity and was used as a measure of theta power. REM sleep latency was defined as the time that elapsed between the onset of the preceding NREM bout to the onset of the REM sleep episode.

Acute sleep deprivation for probing adenosine regulation of sleep homeostasis:

Mice were acutely sleep-deprived (6hr) beginning at the onset of the light cycle (ZT0) using gentle handling. Mice were transferred to a new cage and monitored throughout the sleep deprivation period for signs of sleep (extended periods of inactivity, adoption of sleep posture) by an observer. Mild stimuli were used to prolong wakefulness, including rotating the cage, disassembling or removing the bedding, or placing a nitrile glove in the corner of the cage and gently brushing the animal with the glove (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012).

Electrophysiology:

Whole cell and field recording: Preparation and Recording of Acute NAc Slice: Mice were anaesthetized with isoflurane then decapitated. The brain was rapidly removed and placed in cold cutting solution (low $Ca^{2+}$, high sucrose ACSF [in mM], 84.9 NaCl, 25

$NaHCO_3$, 0.75 $NaH_2PO_4$, 25 Glucose, 0.57 Ascorbic Acid, 0.91 Sodium Pyruvate, 2.5 KCl, 0.25 $CaCl_2$, 4 $MgCl_2$) bubbled continuously with a 95% O, 5% $CO_2$ gas mixture. Sagittal slices 310μm thick were cut and isolated NAc slices were transferred to a bath containing pre-equilibrated recording solution (standard ACSF containing 1mM $MgCl_2$ and 2mM $CaCl_2$) at 30C where they were allowed to recover for 1.5 hours. Recordings were made in standard ACSF supplemented with 100μM picrotoxin to inhibit GABA transmission. Electrode positioning was made based on anatomical markers.

Whole Cell Recording: MSNs in the NAc were targeted based on anatomical location and morphology. Glass electrodes with resistance at 3-5MegaOhms were used. Intrapipette solution (in mM); $K^+$ Gluconate 128, NaCl 20, $MgCl_2$ 1, EGTA 1, $CaCl_2$ 0.3, $Na_2ATP$ 2, NaGTP 0.3, Hepes 10, Qx-314 2.5 to pH 7.3 with KOH, 290-300mOsm. Biocytin (2mg/mL) was included in pipette solution for streptavidin staining. Uncompensated access resistance was monitored throughout the experiment. Signals were filtered at 1KHz and digitized at 5KHz.

Statistical analysis:

SigmaStat was used to perform all statistical analysis. Data are presented as mean ± SEM. Comparisons between two groups were conducted with Student's *t*-test. The difference between groups that had multiple data points were assessed by two-way repeated measures ANOVA. ANOVA was followed by a Tukey's *post hoc* multiple-comparisons test. Statistical significance was defined as $p < 0.05$. Raw data were transformed to Log10 for ANOVA analysis in pain study.

## Chapter 3 Results: Gliotransmission Modulates Baseline Mechanical Nociception

Contributing authors: McIver, S. performed some of the experiments included in this chapter.

### Section 3.1: Overview

Pain is a physiological and adaptive process which occurs to protect organisms from tissue damage and extended injury. Pain sensation beyond injury, however, is a pathological process that is poorly understood. Experimental models of neuropathic pain demonstrate that reactive astrocytes contribute to reduced nociceptive thresholds. Astrocytes release "gliotransmitters" such as D-serine, glutamate, and ATP, which is extracellularly hydrolyzed to adenosine. Adenosine 1 receptor activation in the spinal cord has anti-nociceptive effects on baseline pain threshold, but the source of the endogenous ligand (adenosine) in the spinal cord is unknown. In this study we used a transgenic mouse model in which SNARE-mediated gliotransmission was selectively attenuated (called dnSNARE mice) to investigate the role of astrocytes in mediating baseline nociception and the development of neuropathic pain. Under baseline conditions, immunostaining in the dorsal horn of the spinal cord showed astrocyte-specific transgene expression in dnSNARE mice, and no difference in expression levels of the astrocyte marker, GFAP, or the microglia marker, Iba1, relative to wild-type mice. The Von Frey filament test was used to probe sensitivity to baseline mechanical pain thresholds and allodynia following the spared nerve injury model of neuropathic pain. DnSNARE mice exhibit a reduced nociceptive threshold in response to mechanical stimulation compared to wild-type mice under baseline conditions, but nociceptive thresholds following spared nerve injury (SNI) were similar between dnSNARE and wild-type mice. This study is the

first to provide evidence that gliotransmission contributes to basal mechanical nociception.

**Section 3.2: The dnSNARE transgene is expressed in the spinal cord, is astrocyte specific, and does not induce reactive astrocytes or microglia**

The dnSNARE mice were created by crossing two lines of transgenic mice using the tetracycline regulatory system: in one line of mice, the astrocyte-specific Glial Fibrillary Acidic Protein (GFAP) promoter was used to drive expression of tetracycline transactivator, and in the other line, the tetracycline off promoter was used to drive expression of the enhanced green fluorescent protein (EGFP) and LacZ reporter genes and dominant-negative expression of the cytosolic portion of the synaptobrevin protein (dnSNARE) to attenuate vesicle fusion. This system allows for inducible transgene expression through removal of doxycycline from the diet. Previous studies show astrocyte-selective transgene expression throughout the brain, including the cortex and hippocampus (Halassa, Florian et al. 2009, Deng, Terunuma et al. 2011).

To test for astrocyte-specific transgene expression in the spinal cord, levels L4 to L6 were histologically examined. Immunostaining shows GFAP (glial fibrillary acidic protein) colocalization with the EGFP transgene, whereas the microglial marker Iba1 (ionized calcium-binding adapter molecule 1) and the neuronal marker NeuN do not ($n=3$; EGFP+/GFAP+ cells= $69.2 \pm 7.3\%$; EGFP+/NeuN+ and EGFP+/Iba1+ cells = 0; $P < 0.001$), suggesting that dnSNARE expression in the spinal cord is restricted to astrocytes (Figure 1A, B).

**Section 3.3: dnSNARE mice do not have reactive astrocytes or microglia in the dorsal horn of the spinal cord.**

Because reactive responses in astrocytes and microglia are known to mediate NPP, we quantified levels of reactivity in these cell types prior to injury. Each marker was quantified as percent area of substantia gelatinosa above background staining. We found GFAP (n=3 (WT), n=3 (dnSNARE); P = 0.24) and Iba1 (n=3 (WT), 3 (dnSNARE); P = 0.20) staining was similar in the spinal cords of dnSNARE and WT mice (Figure 1C).

**Section 3.4: dnSNARE mice have increased baseline mechanical nociception**

Since astrocytic expression of dnSNARE causes reduced A1R activation (Halassa, Florian et al. 2009), and stimulation of this receptor in the spinal cord has anti-nociceptive effects on baseline pain (Gong, Li et al. 2010), we hypothesized that dnSNARE mice would have altered basal nociception. We measured mechanical nociception in WT and dnSNARE adult male mice, aged 8-10 weeks, using von Frey filaments, a method in which a constant pressure is applied to the plantar surface of the hind paw and subsequent paw withdrawal threshold is measured. DnSNARE mice exhibit a reduced threshold to mechanical nociception (WT: 1.24 ± 0.13 n=18; dnSNARE: 0.86 ± 0.08 n=21; $p<0.01$), suggesting they have increased baseline sensitivity to pain (Figure 1D). Given that acute pain sensation is a spinal reflex, we anticipate that this effect is mediated by dorsal horn astrocytes. However, we cannot rule out the potential contribution of supraspinal brain regions, such as the rostral ventral medulla or anterior cingulate cortex, where dnSNARE expressing astrocytes are also present (not shown).

Furthermore, it is unlikely that satellite glial cells in the dorsal root ganglion are involved, as GFAP is not highly expressed in these cells before injury (Ohara, Vit et al. 2009).

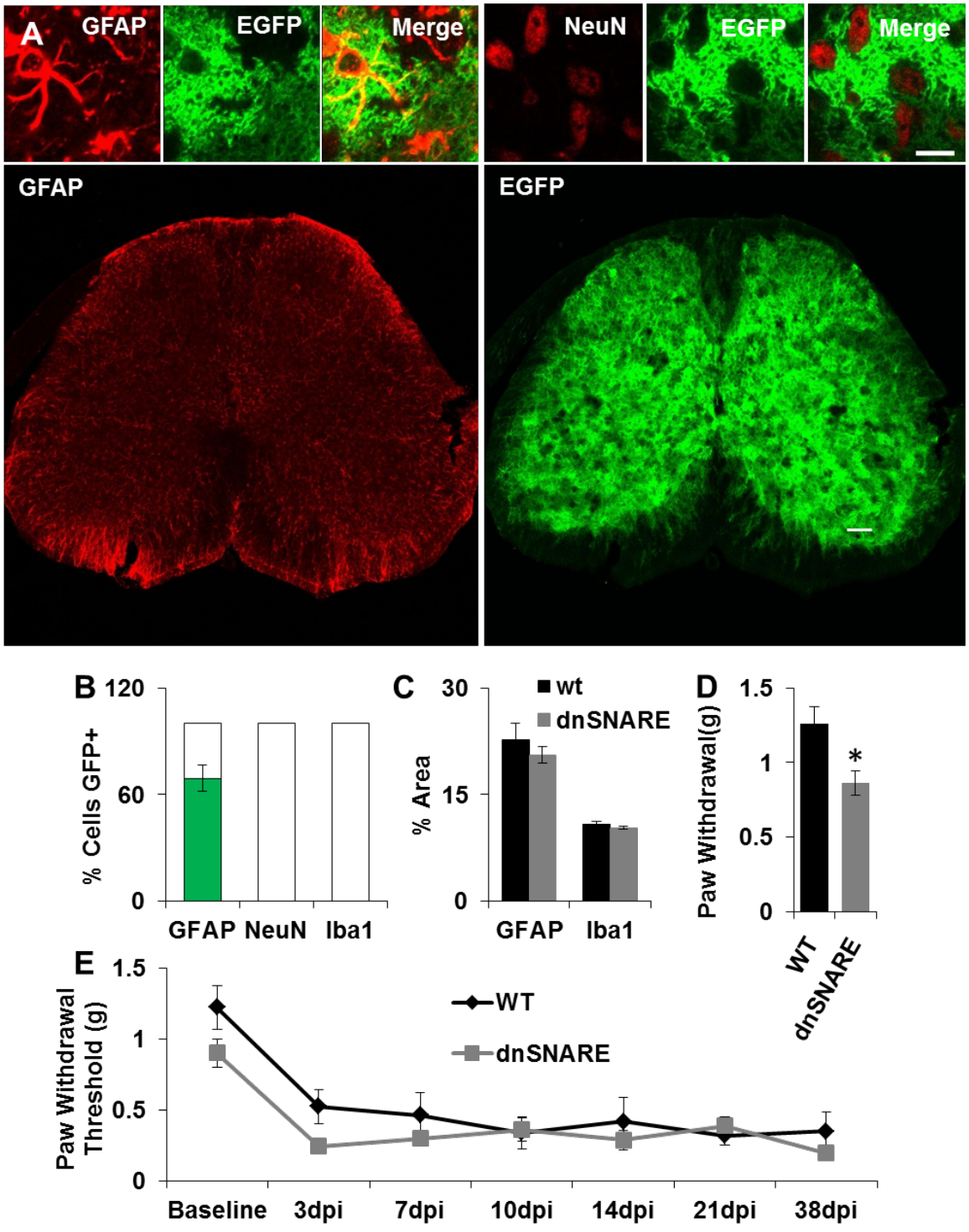

**Figure 1: Astrocyte specific attenuation of gliotransmission causes reduced basal nociception but does not alter NPP.** (A) The lumbar spinal cord exhibits abundant expression of EGFP reporter protein (green) with distinct colocalization with the astrocyte marker, GFAP (red; left), but not with the neuronal marker NeuN (red; right). (B) Quantification of EGFP+ cells reveals that 69.2 ± 7.3% GFAP+ cells were EGFP+ in dnSNARE mice but 0% colocalization was present Iba1+ or NeuN+ cells. No EGFP+ cells were found in WT sections (not shown). (C) dnSNARE expression does not cause reactive astrocytes or microglia as shown by similar GFAP (WT: 22.7 ± 2.4% n=3; dnSNARE: 20.6 ± 1.2% n=3; P = 0.24) and Iba1 (WT: 10.7 ± 0.5% n=3; dnSNARE: 10.3 ± 0.2% n=3; P = 0.20) staining between dnSNARE and WT dorsal horns. (D) DnSNARE mice exhibit a significant reduction in baseline paw withdrawal thresholds compared to WT mice (WT: 1.24 ± 0.13 n=18, dnSNARE: 0.86 ± 0.08 n=21 *P<0.01). (E) DnSNARE and WT mice both exhibit sustained reduction in paw withdrawal threshold after SNI with no significant difference between WT and dnSNARE (WT: n=9 for 3-21dpi, n=7 for 28dpi dnSNARE: n=12 for 3-21dpi, n=7 for 28dpi P = 0.570). Scale bars: 10mm (upper); 100mm (lower). dpi, days post injury.

**Section 3.5: dnSNARE expression does not alter the development of NPP**

Given that baseline nociception is altered in dnSNARE mice, and since these mice are known to exhibit reduced release of ATP, a purine known to play important roles in neuropathic pain, we asked whether astrocytic dnSNARE expression alters the development of NPP. The SNI method of NPP (Decosterd and Woolf 2000, Bourquin, Suveges et al. 2006, Scholz, Abele et al. 2008) was used to test this hypothesis. One day following measurement of baseline nociception, mice were anesthetized and the tibial and peroneal branches of the sciatic nerve were ligated, sparing the sural branch. Animals were allowed to recover from surgery and Von Frey monofilaments were used to test NPP at 3, 7, 10, 14, 21, and 28 days post-injury (dpi). Despite a clear difference in basal pain perception, dnSNARE mice exhibit progressive development of NPP after SNI, similar to WT mice (FIG 1D; WT: n=9 for 3-21dpi, n=7 for 28dpi; dnSNARE: n=12 for 3-21dpi, n=7 for 28dpi; $P = 0.570$).

Altogether, these results suggest that astrocyte gliotransmission is antinociceptive, since attenuated gliotransmission results in increased baseline pain. Despite the role of gliotransmission in controlling baseline pain, dnSNARE mice exhibited normal progression of NPP after SNI. This indicates that gliotransmission serves to alleviate baseline pain, but does not play a significant role in the development of NPP. This suggests the mechanisms involved in NPP development dominate the effects of gliotransmission. Indeed, many neuronal proteins are altered after SNI (Singh, Yaster et al. 2009). Despite the lack of a role in NPP, the fact that attenuated gliotransmission results in reduced mechanical threshold to pain, suggests targeting astrocytes may be one way to treat acute pain or alleviate but not reverse the symptoms of NPP.

## Chapter 4 Results: The Role of Gliotransmission in Nucleus Accumbens Function

Contributing authors: Deng, Q. performed some of the experiments included in this chapter.

### Section 4.1: Overview

For this study, we tested A1R activation and glutamatergic signaling in the NAc of mice with genetic overexpression of a dominant negative SNARE mutation specifically in astrocytes (dnSNARE mouse). Although recordings made from dnSNARE brain slices revealed reduced adenosine tone acting on presynaptic A1Rs, whole cell recordings of MSNs suggested post-synaptic glutamate receptor function was similar to wild type mice. The results of this study support findings in other brain regions that an astrocytic source of adenosine inhibits evoked presynaptic release by A1R activation. However, unique from other brain regions, attenuated gliotransmission in the NAc does not result in altered action potential-independent release or post-synaptic glutamate receptor activity. Given that dnSNARE mice do not exhibit relapse behavior and have reduced adenosine tone in the NAc, a brain region that mediates relapse behavior, an astrocyte source of adenosine tone in the NAc may play a role in mediating cocaine relapse behavior.

### Section 4.2: Expression of the dnSNARE transgene

As previously described, mice with attenuated gliotransmission, dnSNARE mice, were used to test the effect of attenuated SNARE-mediated gliotransmission. We show EGFP reporter expressed in cells in the NAc, surrounding a biocytin loaded patched MSN (Figure 2B), indicative of dnSNARE expression surrounding recorded MSNs.

**Section 4.3: Adenosine tone is reduced in the NAc when gliotransmission is attenuated**

Early findings from the Haydon lab revealed release of gliotransmitters from astrocytes causes NMDAR-mediated $Ca^{2+}$ increases in neurons (Parpura, Basarsky et al. 1994). More recently, the lab has shown that dnSNARE mice have altered basal synaptic properties and neuronal plasticity (Pascual, Casper et al. 2005). This has been shown to result in decreased adenosine tone and attenuated long-term potentiation (LTP) in hippocampal slices (Pascual, Casper et al. 2005) and a reduction in surface expression of NR2A and B subunits of the NMDA receptor (Fellin, Halassa et al. 2009). More recent findings in the lab have revealed these mice have altered sensitivity to addictive substances, including cocaine (Turner, Ecke et al. 2013).

Pre-synaptic A1R activation inhibits glutamate release in the NAc (Uchimura and North 1991). Studies performed in the hippocampus have shown that an astrocytic source of tonic adenosine is the cause of inhibited release in this brain region (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012). Using similar methods in the NAc, it was found that dnSNARE mice have reduced adenosine tone, compared to wild type mice. Application of 200nM CPT (A1R antagonist) resulted in an increase in the normalized fEPSP amplitude in wild type mice, but this effect was significantly reduced in dnSNARE mice (Figure 2C; wt: 1.18+/-0.04, n=5; dnSNARE: 1.05+/-0.03%, n=5; *$p<0.05$, t-test). These data suggest that a source of A1R activation is missing in dnSNARE mice and pre-synaptic inhibition of evoked glutamate release in the NAc is reduced. To control for the possibility that the observed effect was due to a decreased number of A1Rs the A1R agonist, CCPA, was tested at increasing doses in both dnSNARE and wild type mice. No

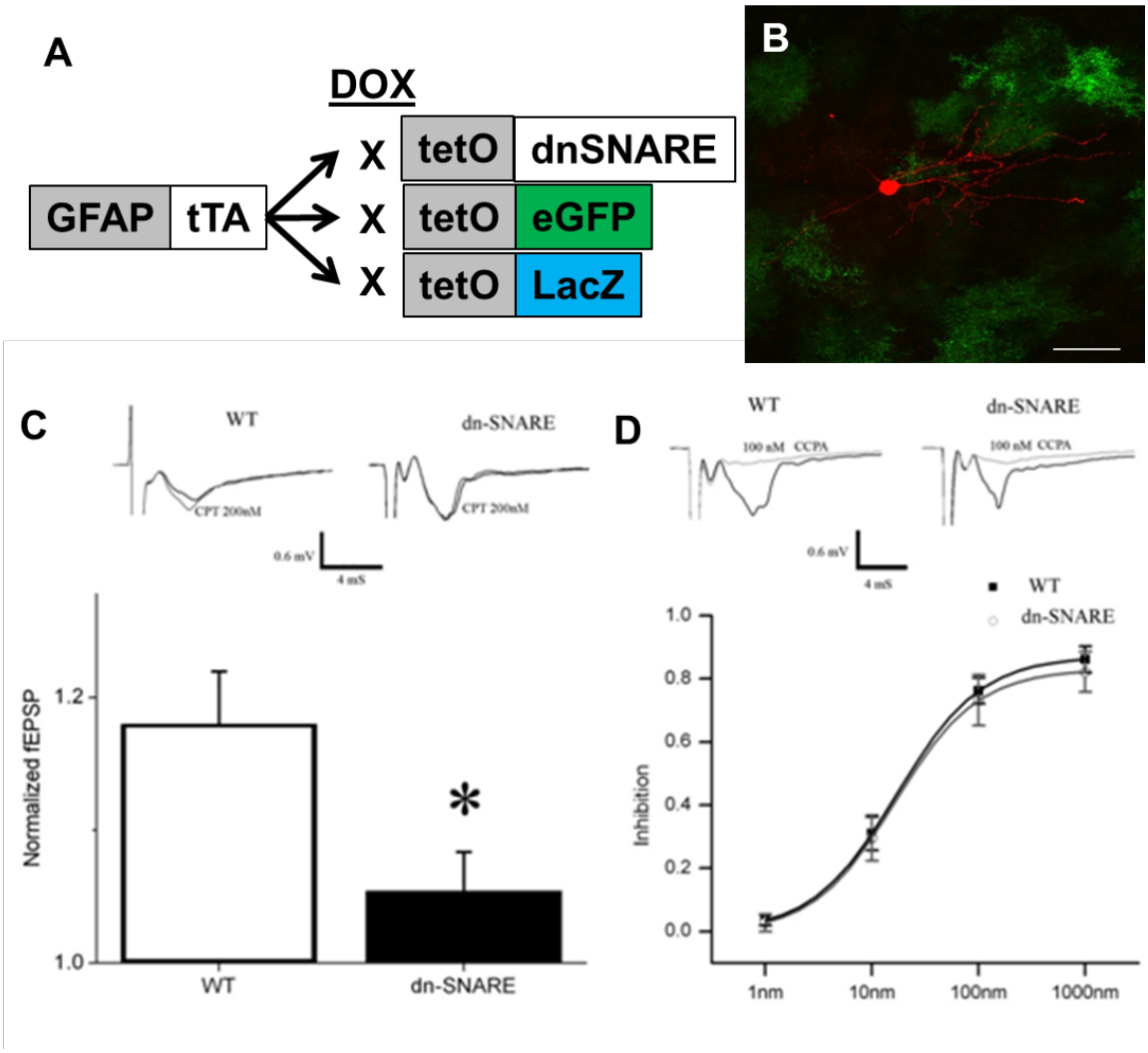


**Figure 2: Adenosine tone is reduced in the NAc when gliotransmission is attenuated.** (A) tTA expression is driven by the GFAP promoter to ensure astrocyte specific expression. The dnSNARE transgene and eGFP and LacZ reporter genes are driven by tetO when bound to tTA, which is blocked by doxycycline. (B) Labeled MSN in the NAc surrounded by eGFP expressing cells. Scale bar= 50 microns. (C) Representative fEPSP traces from NAc slices. Wt and dnSNARE traces before (black line) and after 200nM CPT (grey line) (top). Histogram (below) shows dnSNARE mice have decreased sensitivity to 200nM CPT, compared to wt, which show significantly increased fEPSP amplitude. (D) Representative fEPSP traces from wt and dnSNARE before (black line) and after CCPA application (grey line) (top). Dose-response curve (bottom) of inhibitory action of CCPA. Data are represented as mean +/- SEM.

significant differences in the dose-response curves of A1R agonist application were found between wild type and dnSNARE mice (Figure 2D; Two way RM ANOVA; n.s.), suggesting overall receptor sensitivities were similar. These results suggest that pre-synaptic A1R induced inhibition of stimulated glutamate release in the NAc is mediated by an astrocytic source of adenosine tone.

A1Rs are present on neurons both pre- and post-synaptically, and modify neuronal function by inhibiting pre-synaptic release and decreasing excitability, respectively (Harvey and Lacey 1997, Fellin, Pascual et al. 2004, Deng, Terunuma et al. 2011). We therefore sought to determine whether the attenuated adenosine tone in dnSNARE mice had an effect on MSN passive membrane properties and excitability. To this end, MSNs were recorded in current clamp and current was injected from -400pA to +400pA in 50pA steps. No significant difference was found when the voltage/current data were plotted for wild type and dnSNARE mice (Figure 3B; wt, n=3; dnSNARE, n=3; Two Way RM ANOVA: n.s.). Furthermore, resting membrane potential was not significantly different between wild type and dnSNARE mice (wt: -75.4+/-2.5mV; dnSNARE: -74.6+/-0.7mV; t-test: n.s.). These data suggest that attenuated gliotransmission does not alter passive membrane properties governing the resting membrane potential of MSNs.

To further assess intrinsic membrane properties, we measured MSN excitability and found that when surrounding astrocytes had attenuated gliotransmission, NAc MSNs had increased excitability, characterized by increased number of action potentials per current injection (200pA injection: Figure 3C; WT: 1.5+/-0.2, n=9; dnSNARE: 5+/-0.5, n=4). Accordingly, dnSNARE mice had decreased latency to initial spike onset (Figure

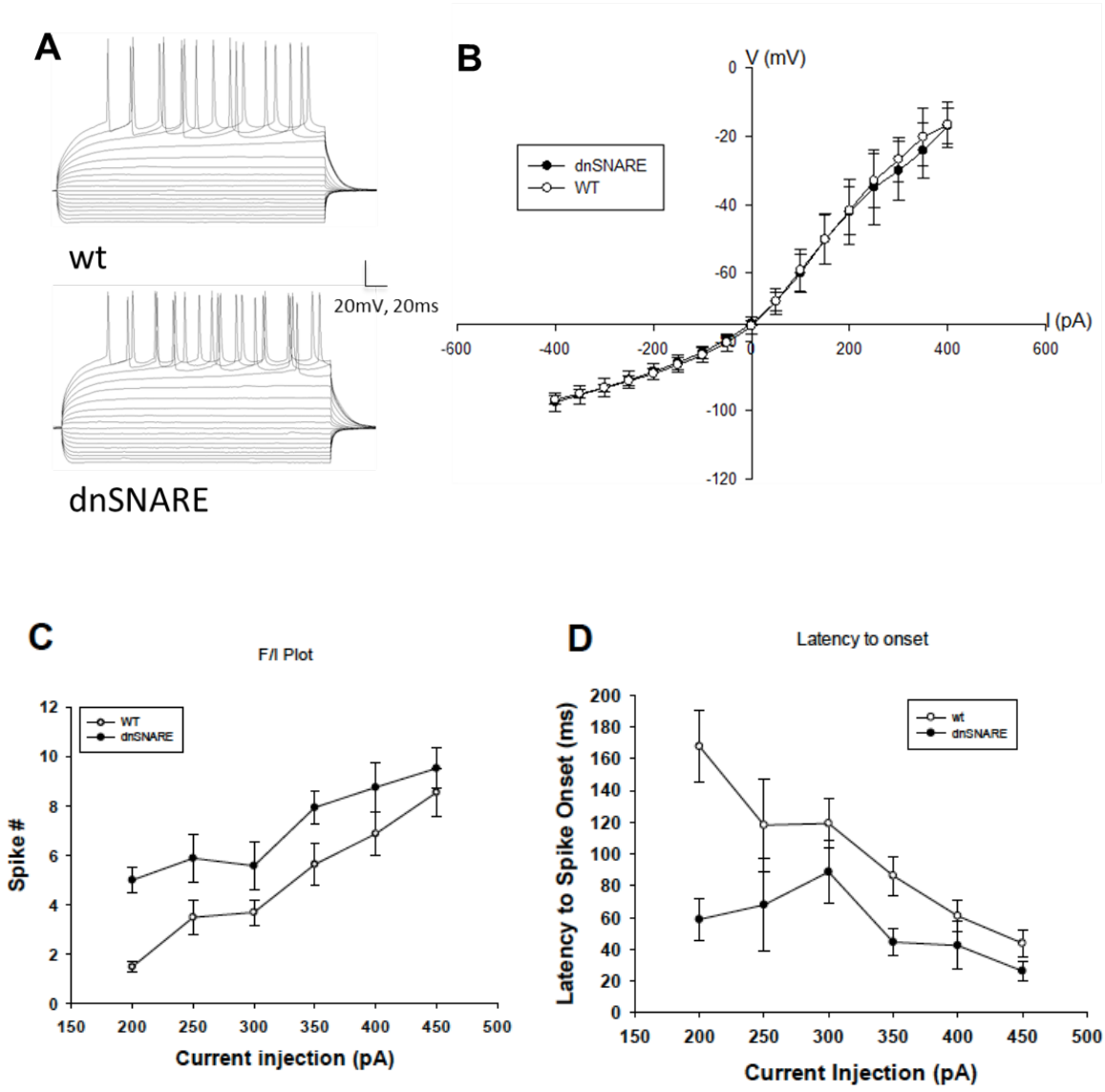


**Figure 3: Attenuated gliotransmission results in increased excitability in the NAc.** (A) representative traces from wt and dnSNARE MSNs current clamped from -400 to +400 pA. (B) Mean and standard deviation from experiments described in figure 2A (dnSNARE, n=3; wt, n=3). (C) F/I plot showing mean+/-SEM of action potential number per current injection. dnSNARE have increased numbers of action potentials at lower current injections. (D) Latency to initial action potential (in ms) in dnSNARE and wt mice. dnSNARE have reduced latency to action potential. Data are represented as mean+/-SEM.

3D; 200pA injection: WT: 167.8+/-22.5ms; n=9; dnSNARE: 58.8+/-12.8ms, n=4). These data suggest that astrocyte gliotransmission decreases excitability, possibly through an A1R-dependent mechanism.

**Section 4.4: Baseline synaptic activity in the NAc is unaffected by attenuated gliotransmission**

One example of how astrocytes control neuronal excitability in the NAc is through NMDAR function (Fellin, D'Ascenzo et al. 2007). Specifically, astrocyte derived adenosine increases NMDAR function in the hippocampus. Since dnSNARE mice have reduced adenosine tone in the NAc (Figure 2), we tested the effect of the dnSNARE manipulation on glutamatergic transmission in the NAc. MSNs were voltage clamped at negative 65mV and perfused with TTX to prevent action potential dependent neurotransmission. Action potential independent mini excitatory postsynaptic currents(mEPSCs) were recorded and frequency and amplitude were quantified. No significant difference in mEPSC amplitude was found between wt and dnSNARE mice (Figure 4B, D: wt: 18.7+/-1.7pA, n=5; dnSNARE: 23.2+/-3.8pA, n=4; t-test: n.s.), suggesting levels of post synaptic glutamate receptor activation in NAc MSNs were not altered by attenuated gliotransmission. These data suggest that the post-synaptic AMPA receptor density, which makes up the majority of the mEPSC amplitude, is not changed in dnSNARE mice. Furthermore, we found no significant difference between wild type and dnSNARE mEPSC inter-event-intervals (Figure 4C,E: wt: 293.9+/-55.4ms, n=5; dnSNARE: 339.4+/-92.4, n=4), indicating that there is no difference in spontaneous, action potential-independent release of transmitter onto MSNs.

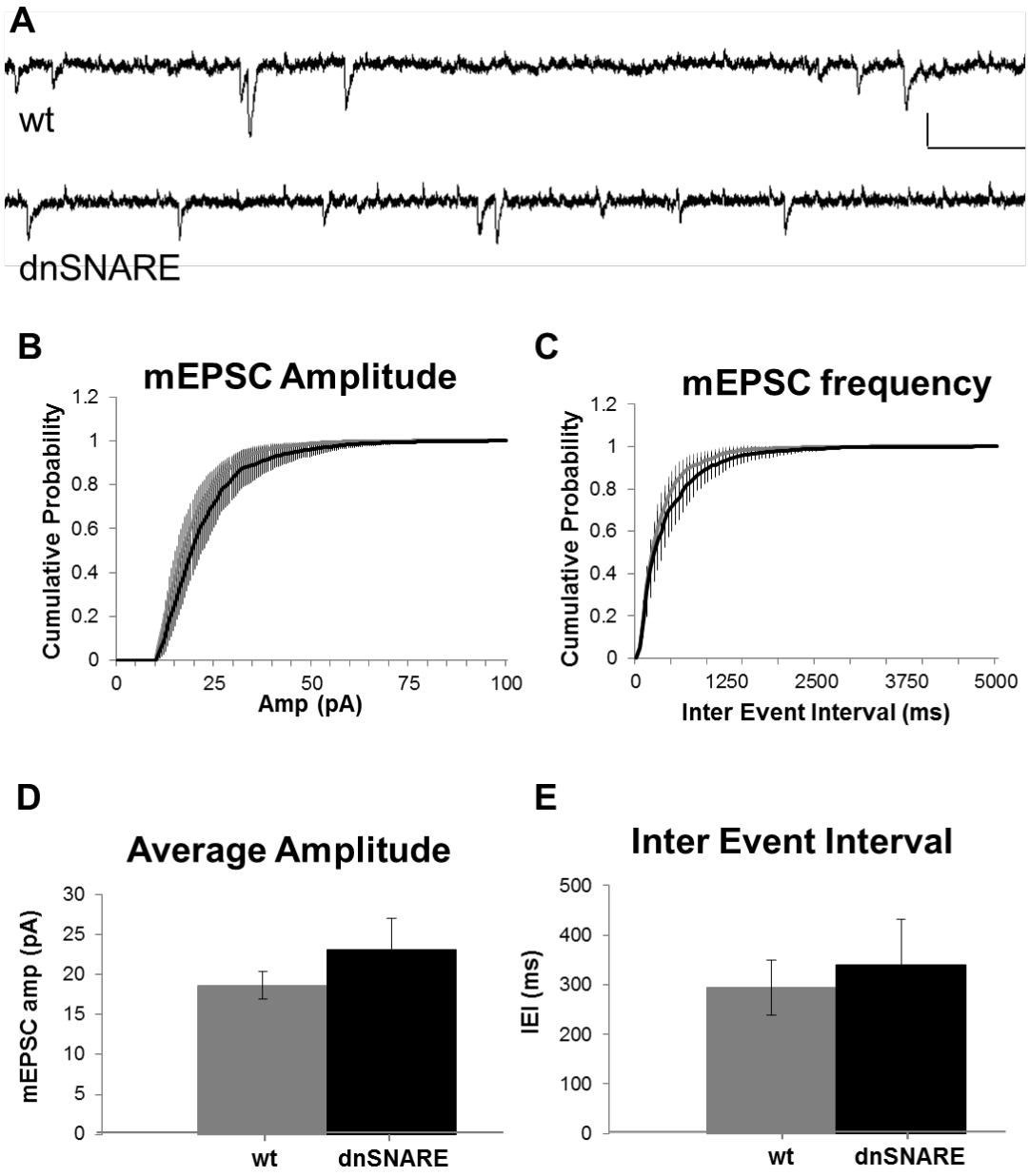


**Figure 4: Baseline synaptic activity in the NAc is unaffected by attenuated gliotransmission.** (A) representative traces of wt (top) and dnSNARE (bottom) voltage clamped (-65mV) MSNs. Scale bar, 20pA, 100ms, wt: n=5, dnSNARE: n=4. (B) Cumulative probability of mEPSC amplitude of wt (grey line) and dnSNARE (black line). (C) Cumulative probability of mEPSC frequency, represented as inter event interval, of wt (grey line) and dnSNARE (black line). (D) Average mEPSC amplitude for wt and dnSNARE MSNs. (E) Average inter event interval (ms) between mEPSCs in wt and dnSNARE MSNs.

**Section 4.5: PFC-evoked AMPA/NMDA ratios in MSNs are unchanged when gliotransmission is attenuated**

The NAc receives input from many different brain regions, including the PFC, hippocampus, amygdala, and the VTA. To observe the post synaptic response of MSNs specifically from PFC afferents, these afferents were visualized in sagittal sections and stimulated while post synaptic evoked EPSCs were recorded from a +40mV voltage-clamped MSN. After stable EPSCs were obtained, APV was applied and the APV resistant (AMPA) component was subtracted from the baseline value to isolate the NMDA component. As an internal control for variability of evoked AMPA and NMDA responses, the ratio of AMPA to NMDA responses were taken to allow for reliable comparison across genotype. There was no significant difference between the PFC evoked AMPA/NMDA ratios recorded from MSNs in the NAc of dnSNARE vs. wild type mice (Figure 5; wt: 2.3+/-5, n=6; dnSNARE 2.3+/-4, n=5). Combined with the result that there is no change in mEPSC amplitude (suggesting no change in AMPAR function), these data suggest that NMDAR function is not altered by the dnSNARE method of attenuated gliotransmission in the NAc. Furthermore, this finding suggests a distinct role for gliotransmission in regulating NMDAR function in the NAc, from that of the hippocampus, where genetically expressing dnSNARE in astrocytes reduces NMDAR function.

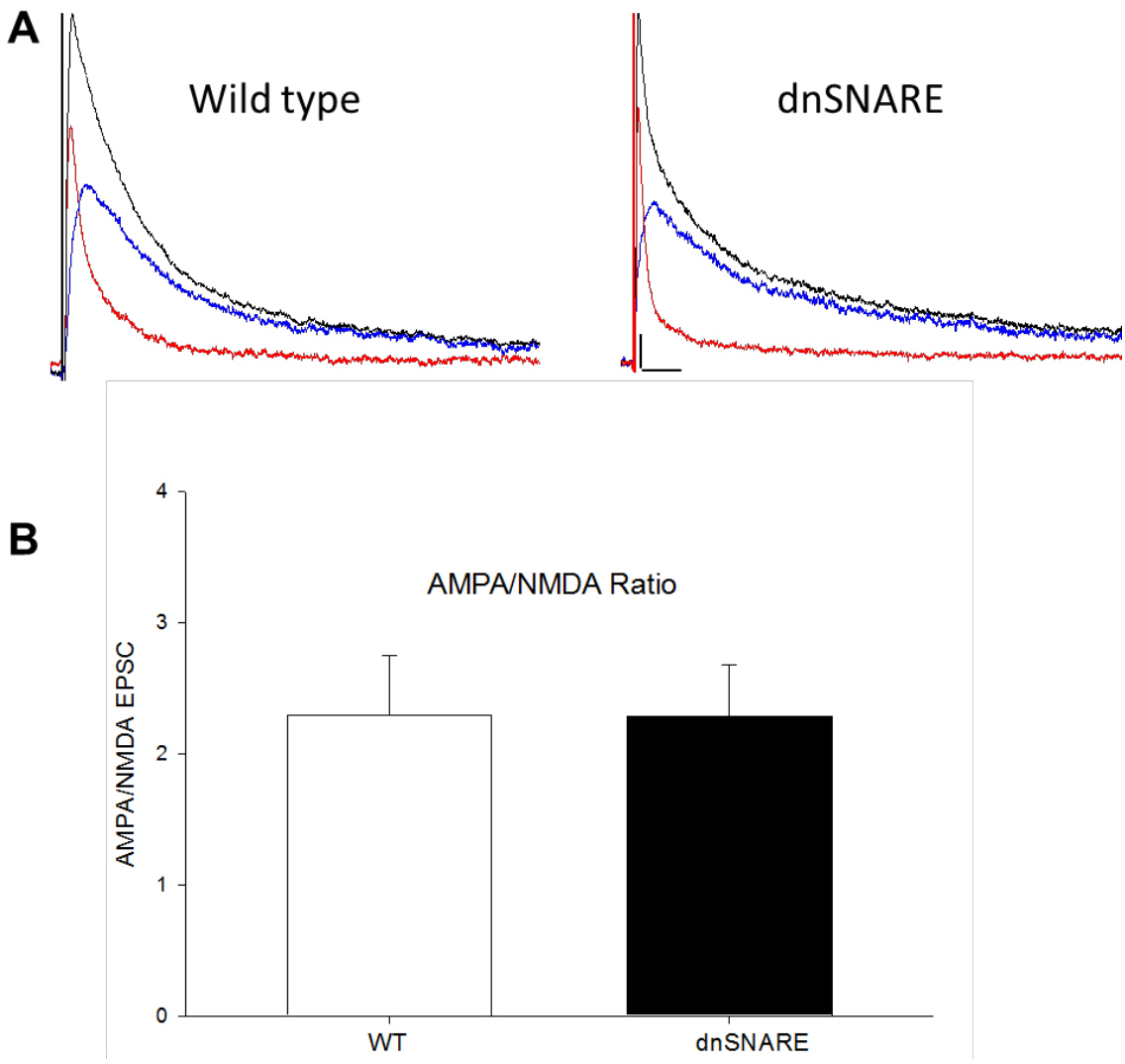


**Figure 5: PFC evoked AMPA/NMDA ratios are unchanged when gliotransmission is attenuated.** (A) Representative traces from dnSNARE and wild type PFC evoked EPSCs. Baseline (black trace) prior to APV application represents the dual component evoked EPSC. The AMPA component is represented (red trace) as the APV insensitive component. The NMDA component (blue trace) is represented as the APV insensitive component subtracted from the dual EPSC. (B) Histograms representing average AMPA/NMDA ratio of peak currents from pharmacologically isolated portions of the EPSC from dnSNARE and wild type mice (WT, n=6; dnSNARE, n=5).

Altogether, these results suggest that gliotransmission contributes to adenosine tone in the NAc, but does not significantly influence presynaptic release of glutamate or post-synaptic function of glutamate receptors. Attenuated gliotransmission increases firing rate elicited by lower current injections (200pA), but it is not clear whether this is tied to the attenuated adenosine tone also present in the NAc of dnSNARE mice.

## Chapter 5 Results: Astrocyte $IP_3/Ca^{2+}$ Signaling Selectively Regulates Theta Rhythm and REM Sleep.

Contributing authors: Blutstein, T. performed some of the experiments included in this chapter.

### Section 5.1: Overview

Our lab has shown that the gliotransmitter, adenosine, is involved in sleep homeostasis and NREM sleep in mice (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012). Considering that $Ca^{2+}$ signaling is required for neurotransmission, and there is extensive evidence that it is required for gliotransmission (Porter and McCarthy 1997, Araque, Sanzgiri et al. 1998, Buzsàki 2002, Hua, Malarkey et al. 2004, Kreft, Stenovec et al. 2004, Chen, Wang et al. 2005, Pryazhnikov and Khiroug 2008, Paukert, Agarwal et al. 2014), we asked whether we could replicate the dnSNARE phenotype of impaired sleep homeostasis in a mouse model of disrupted astrocytic $IP_3/Ca^{2+}$ signaling. We overexpressed a Venus tagged $IP_3$ 5'phosphatase (VIPP) transgene selectively in astrocytes to enhance the metabolism of $IP_3$ to $IP_2$ in order to attenuate $IP_3$-mediated mediated $Ca^{2+}$ release in this cell type. To our surprise, VIPP mice have normal NREM sleep, compared to wild type mice, but have increased REM sleep and theta power. The results of this study suggest a previously unrecognized and unique role for astrocytic $IP_3/Ca^{2+}$ signaling in neuromodulation, independent of SNARE mediated release from astrocytes. Moreover, these results demonstrate the importance of the astrocyte, a glial cell sub-type, in controlling sleep states and brain rhythms.

## Section 5.2: The VIPP reporter β-Gal is expressed in astrocytes, but not neurons throughout the brain

We used molecular genetics to over-express $IP_3$ 5' phosphatase specifically in astrocytes, thus reducing $IP_3$-dependent $Ca^{2+}$ release from the ER exclusively in this cell type (Schmitt et al., in prep). The transgene was expressed in an inducible manner using the Tet Off system (Figure 6b). The overexpressed $IP_3$ 5' phosphatase was fused with a Venus fluorescent protein reporter, making a fluorescent tag present wherever the transgene is expressed. Another reporter gene, Lac-Z, was co-expressed with the VIPP transgene. The Lac-Z gene product, β-Galactosidase (β-Gal), was stained and bright field images were obtained to show regional expression. Relevant brain regions in sleep regulation, such as the pons, hippocampus, thalamus, cortex, and basal forebrain (Mothet, Pollegioni et al. 2005, McCarley 2007), all show reporter expression (Figure 6a). To determine cell-type specific expression, antibodies against β-Gal, GFAP, and Neu-N were used to fluorescently label these proteins (Figure 6c). Co-localization was quantified in the hippocampus where 38.3±13.1% of GFAP+ cells were also β-Gal+, whereas none of the Neu-N expressing cells were β-Gal+ (Figure 6c,d), supporting astrocyte specific transgene expression.

## Section 5.3: The baseline percent time spent in REM sleep is increased in mice with attenuated $IP_3/Ca^{2+}$ signaling in astrocytes

Cholinergic transmission regulates REM sleep and astrocytes have acetylcholine receptors that elicit calcium transients when stimulated (Nugent, Penick et al. 2007, Takata, Mishima et al. 2011, Araque, Carmignoto et al. 2014). Intracellular calcium

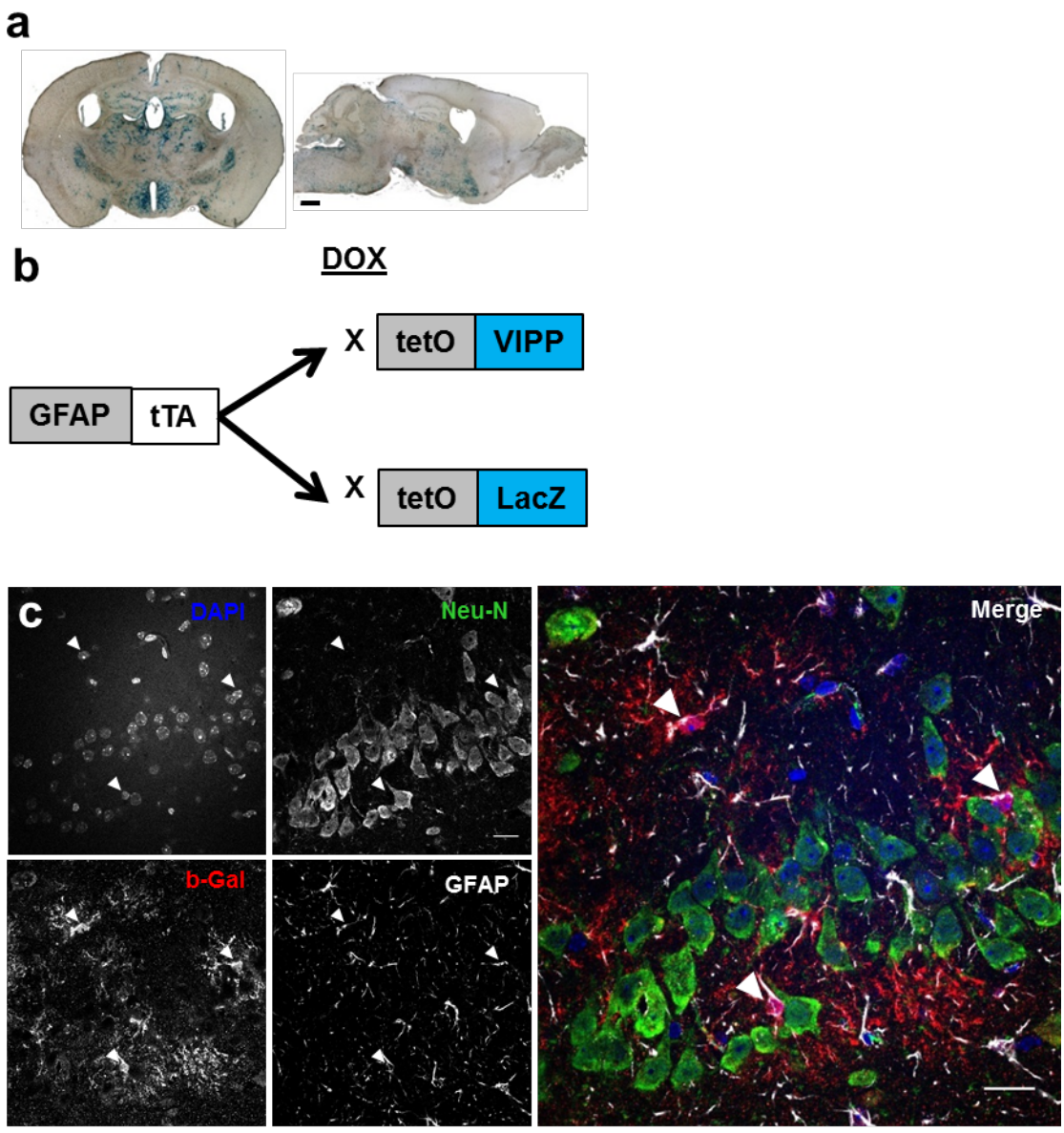
a
b
DOX
X
tetO
VIPP
GFAP
tTA
X
tetO
LacZ
c
DAPI
Neu-N
Merge
b-Gal
GFAP
d
100
50
0
% Cells β-Gal+
GFAP
Neu-N

**Figure 6: VIPP transgene expression is brain wide and astrocyte specific.** (A) VIPP expression represented by β-Gal labeled cells. Scale bar: 100μm. (B) tTA expression is driven by the GFAP promoter to ensure astrocyte specific expression. The VIPP transgene and LacZ reporter genes are driven by tetO when bound to tTA, which is blocked by doxycycline. (C) DAPI labeled nuclei within GFAP and Neu-N fluorescently labeled cells as well as β-Gal expression are shown as 4 greyscale panels (left) with the overlay of all channels (right). Scale bar 10μm. (D) Quantification of co-localization of β-Gal with GFAP and Neu-N cells (wild type, n=3; VIPP, n=3; values represent mean and standard deviation).

signaling is the main mechanism by which astrocytes respond to input because these cells are not electrically excitable. Stimulation of (Gq coupled) G-protein coupled receptors (GPCRs) on astrocytes can induce an elevation of $IP_3$, which in turn stimulates the release of $Ca^{2+}$ from $IP_3$-sensitive ER $Ca^{2+}$ stores. Activation of astrocytes in this way influences neuronal function (Schmitt et al., in prep), however, the role of $Ca^{2+}$ in gliotransmission is controversial (Fiacco, Agulhon et al. 2007, Petravicz, Fiacco et al. 2008, Wang, Smith et al. 2013). We asked whether $Ca^{2+}$-dependent gliotransmission affects brain wide oscillations involved in discrete vigilance states. EEG/EMG recordings were taken from wild type and VIPP mice and vigilance states were assigned based on EEG frequency and EMG power. NREM is dominated by high amplitude, low frequency (0.5-4Hz) delta waves, whereas REM sleep is dominated by lower amplitude, higher frequency (5-8Hz) theta activity. Low EMG activity is present during both NREM and REM activity (Figure 8a,b).

The baseline percent time spent in REM sleep is increased in mice with attenuated $IP_3/Ca^{2+}$ signaling in astrocytes (Figure 7). This effect was most apparent during the light phase (subjective nighttime), with a corresponding increase in number of REM bouts (Figure 7d, VIPP=63.75±1.6, wild type=56.5±2.0; $P<0.01$). REM bout duration was

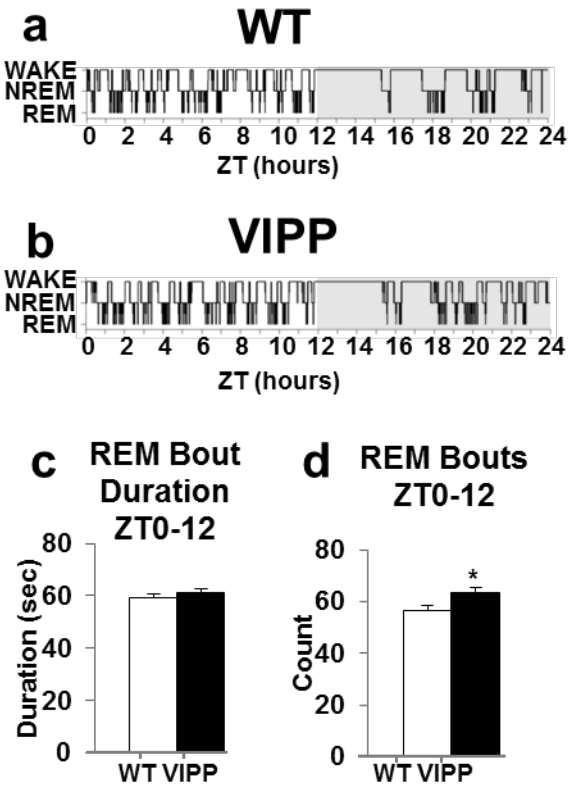


**Figure 7: Mice that overexpress Venus tagged $IP_3$ 5' phosphatase spend more time in REM sleep.** (A) Representative example of a hypnogram from a wild type mouse. (B) Representative example of a hypnogram from a VIPP mouse. (C) Wild type and VIPP mice had similar REM bout durations during the light phase. (D) VIPP mice entered REM sleep more frequently than wild type mice during the light phase (values represent mean and standard error (SEM) for each group, **$p<0.01$, t-test).

unchanged in mice with attenuated $IP_3/Ca^{2+}$ signaling (Figure 7c). VIPP mice spent significantly more time in REM sleep during the first half of the light phase and the second half of the dark phase (Figure 8e, ZT0-6; VIPP: 9.6±0.3%, wild type: 8.8±0.3%; $P<0.05$; ZT18-24; VIPP: 4.9±0.4%, wild type: 3.9±0.4%; $P<0.05$). During the second half of the light phase, when VIPP mice spent a greater percentage of time in REM sleep, compared to wild type (Figure 8e, ZT6-12; VIPP: 8.6±0.4%, wild type: 6.8±0.3% recording time; $P<0.01$), they spent significantly less time in wake (Figure 8c, ZT6-12; VIPP: 34.5±1.3%, wild type: 39.8±2.0%; $P<0.05$). Throughout the entire light/dark cycle, VIPP and wild type mice spent similar time in NREM (Figure 8d).

**Section 5.4: Attenuated astrocytic $IP_3$-dependent $Ca^{2+}$ signaling does not result in a narcoleptic phenotype.**

Narcoleptic human patients exhibit excessive daytime sleepiness and uncontrollable sleep onset at inappropriate times. They spend more time in REM sleep and exhibit decreased latency to enter REM sleep after sleep onset. In some cases, direct wake to REM transitions occur. Since VIPP mice show an increase in theta power and time spent in REM sleep, REM sleep onset latencies and wake to REM transitions during the subjective day (the dark phase) were measured in wild type and VIPP mice. There were no significant differences between the wild type and VIPP mice in transitions from one vigilance state to the next. Specifically, neither genotype exhibited direct wake to REM transitions in the dark phase (Figure 9a). The latency to REM onset from NREM onset was also assessed and there was no significant difference between wild type and VIPP mice (Figure 9b; wild type: 130.1±11.3s; VIPP: 116±7.4s). Thus, it is not likely that the increased theta power and REM sleep phenotype is tied to a narcoleptic

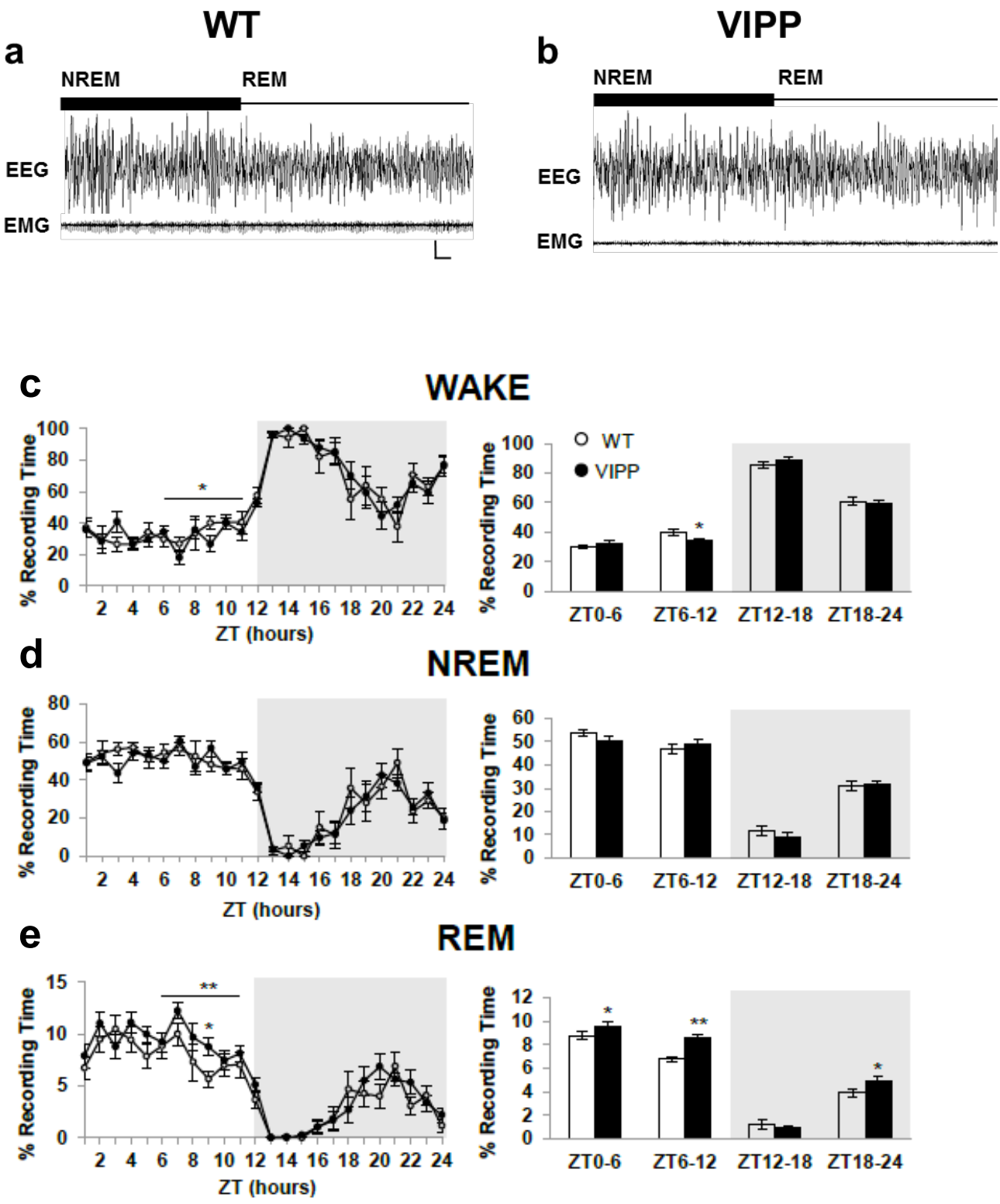


**Figure 8: VIPP expression increases time spent in REM and decreases time spent in wake.** (A) Representative trace of WT EEG and EMG. (B) Representative trace of VIPP EEG and EMG. (C) Percent recording time spent in wake. VIPP mice spent significantly

less time in wake during ZT 6-12 (data is binned by hour and each point represents the mean ± SEM for each group. Left: *p<0.05, Two-way RM ANOVA; right: *p<0.05, t-test). (D) Percent recording time spent in NREM. VIPP and wild type mice spent the same amount of time in NREM (data is binned by hour and each point represents the mean and SEM for each group, Two-way RM ANOVA: n.s.). (E) Percent recording time spent in REM. VIPP mice spent significantly more time in REM sleep during the light cycle and the second half of the dark cycle (data is binned by hour and each point represents the mean and SEM for each group. Left: **p<0.01, Two way RM ANOVA, ZT9: Tukey's post hoc test: p<0.05; right: *p<0.05, **p<0.01, t-test). Data are represented as mean ± SEM.

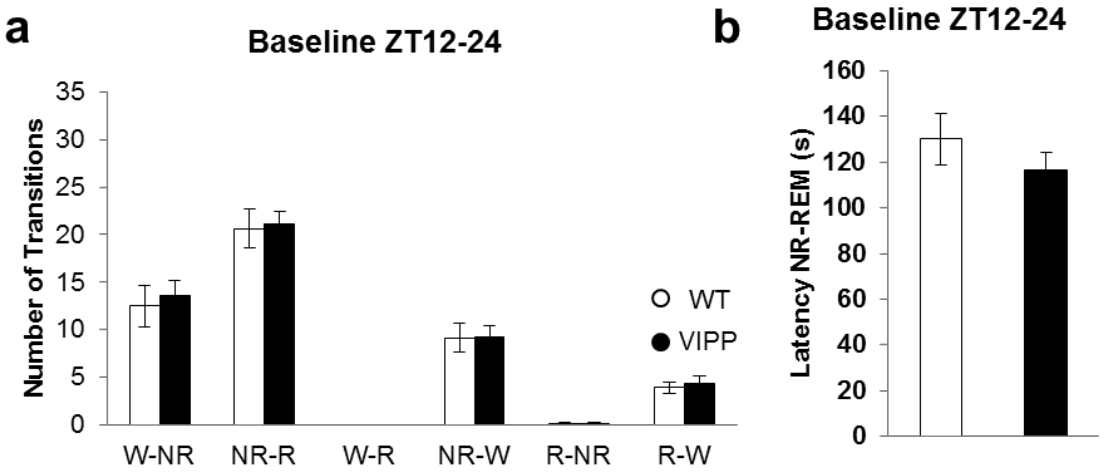


**Figure 9: VIPP mice do not exhibit a narcoleptic phenotype.** (A) Number of transitions from vigilance states in the dark phase. There were no significant differences in transitions from any one state to another. Specifically, direct wake to REM transitions did not occur in either genotype. (B) Latency to REM onset from NREM onset in the dark phase. There was no significant difference between wild type and VIPP mice in the latency to REM onset from NREM in the dark phase.

mechanism, and REM sleep latency is likely not modulated by astrocytic $IP_3/Ca^{2+}$ signaling.

**Section 5.5: VIPP mice have increased theta power across all vigilance states.**

Theta power is increased in VIPP mice. Recent findings in the hippocampus show that attenuated $IP_3$-dependent $Ca^{2+}$ signaling in astrocytes causes a reduction in theta-induced long-term potentiation (Schmitt et al., unpublished). An enhanced EEG theta rhythm was recorded in VIPP mice (Figure 10). This effect was present during wakefulness, NREM and REM sleep. The most robust effect occurred during REM sleep, where theta is the dominant frequency in the power spectrum. However, theta is also a prominent frequency during active stimulus processing during wakefulness (Colgin 2013). In order to assess a time of day dependent effect, the baseline day was broken down into the light and dark phase and power spectra were analyzed for each vigilance state within these time bins. During the light phase, VIPP mice exhibited enhanced power within the theta frequency range across both NREM and REM sleep (Figure 10a; WAKE: NS; NREM: $p<0.05$ at 3.9-4.1, 5.3, 5.8-6.3Hz; REM: $p<0.05$ at 5.4-8.1Hz). When theta power (5-8Hz) was averaged across all frequencies within range and compared between genotypes, VIPP mice had significantly greater theta power during REM sleep (inset: $p<0.05$; wild type: 3.5±0.5; VIPP: 5.0±0.4), compared to wild type mice. During the dark phase, VIPP mice exhibited enhanced theta power across all vigilance states (Figure 10b; WAKE: $p<0.05$ at 7.1-8.1Hz; NREM: $p<0.05$ at 3.9Hz; REM: $p<0.05$ at 6.1-8.1Hz). When theta power was averaged and compared between genotypes, VIPP mice had significantly greater power during REM sleep ($p<0.01$; wild type: 3.7±0.6; VIPP:

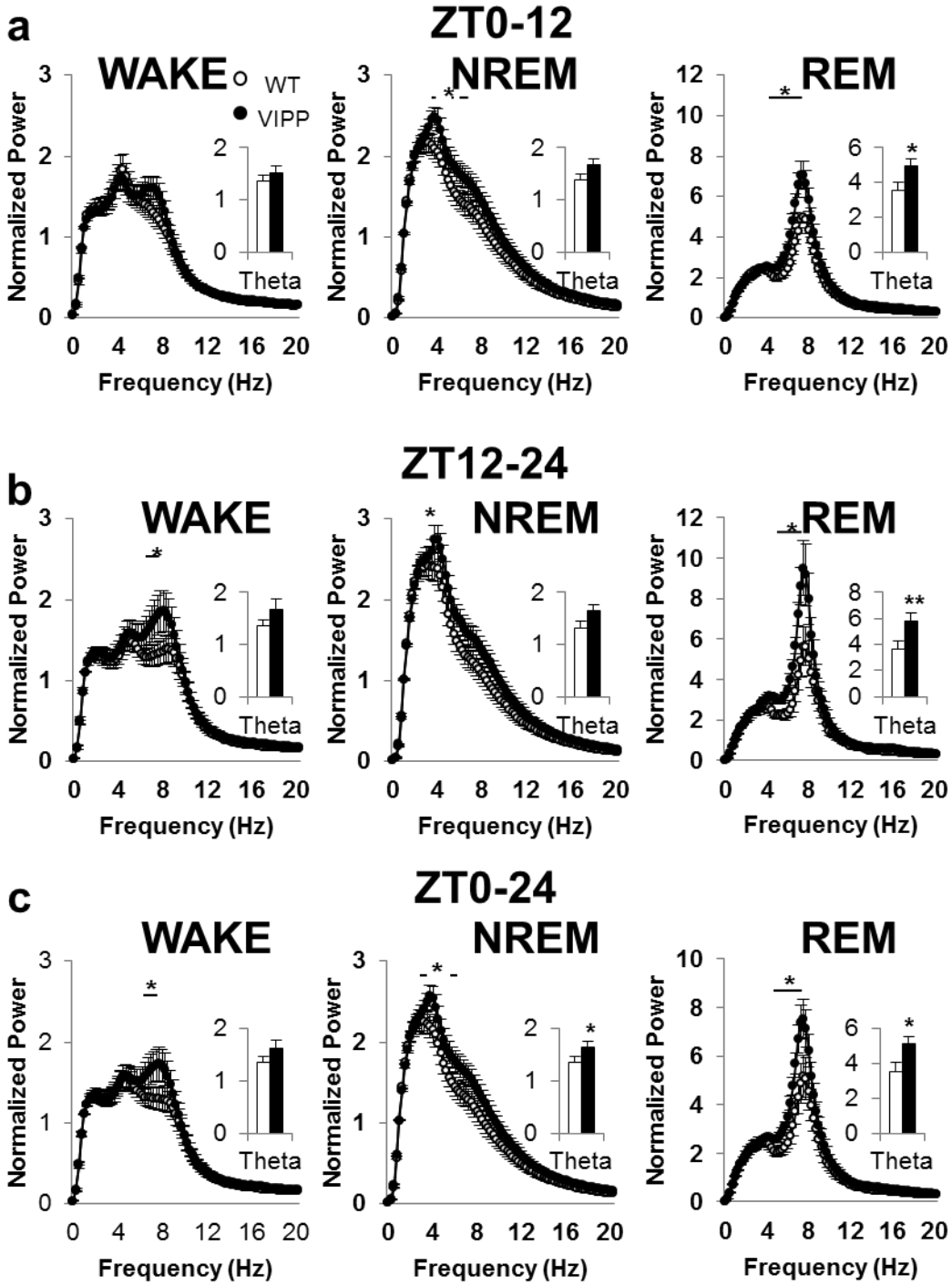


**Figure 10: VIPP mice exhibit increased theta power across all vigilance states compared to wild type mice.** (A) Light cycle FFT data representation of EEG power. Theta power is enhanced in VIPP mice during NREM and REM sleep, inset shows average theta (5-8Hz) power (NREM: Two-way RM ANOVA: *p<0.05; inset: n.s.; REM: Two-way RM ANOVA: *p<0.05; inset, t-test: *p<0.05; Wake: Two-way RM ANOVA: n.s.). (B) Dark cycle FFT data representation of EEG power. Theta power was enhanced in VIPP mice during WAKE, NREM and REM. (WAKE: Two-way RM

ANOVA: *p<0.05; inset: n.s.; NREM: Two-way RM ANOVA: *p<0.05; inset: n.s.; REM: Two-way RM ANOVA: *p<0.05; inset, t-test: **p <0.01). (C) EEG power over the entire 24hr recording period. VIPP mice had increased theta power across all vigilance states (WAKE: Two-way RM ANOVA: *p<0.05; inset: n.s.; NREM: Two-way RM ANOVA: *p<0.05; inset: t-test, *p<0.05; REM: Two-way RM ANOVA: *p<0.05; inset: t-test, *p<0.05). Data are represented as mean ± SEM.

5.8±0.6). When a power analysis was performed on the entire twenty-four hour baseline day, a similar pattern emerged, where VIPP mice exhibited enhanced theta power (Figure 10c; WAKE: p<0.05 at 7.1-8.1Hz; NREM: p<0.05 at 3.6-4.1, 5.8-6.3Hz; REM: p<0.05 at 5.9-8.1Hz). Theta power is significantly higher in VIPP mice when averaged for NREM (p<0.05; wild type: 1.4±0.1; VIPP: 1.7±0.1) and REM sleep (p<0.05; wild type: 3.6±0.5; VIPP: 5.1±0.4).

**Section 5.6: $IP_3/Ca^{2+}$ signaling affects sleep in a discrete pathway from prevention of vesicular-mediated release in astrocytes.**

We have previously published that mice with attenuated gliotransmission (dnSNARE mice) have reduced slow wave activity (SWA) delta power (0.5-4.0Hz) and low frequency (0.5-1.5Hz) slow wave activity (lf-SWA), compared to wild type mice (Halassa, Florian et al. 2009). This phenotype occurs at baseline and is exaggerated after sleep deprivation, where SWA and lf-SWA power increases in wild type but stays low in dnSNARE mice. Given that neurotransmitter release relies on $Ca^{2+}$ signaling, and that there is mounting evidence for $Ca^{2+}$-dependent gliotransmission (Porter and McCarthy 1997, Araque, Sanzgiri et al. 1998, Buzsàki 2002, Hua, Malarkey et al. 2004, Kreft, Stenovec et al. 2004, Chen, Wang et al. 2005, Pryazhnikov and Khiroug 2008, Paukert, Agarwal et al. 2014), we asked whether mice with overexpression of $IP_3$ 5' phosphatase specifically in astrocytes had a similar phenotype to mice with attenuated SNARE-

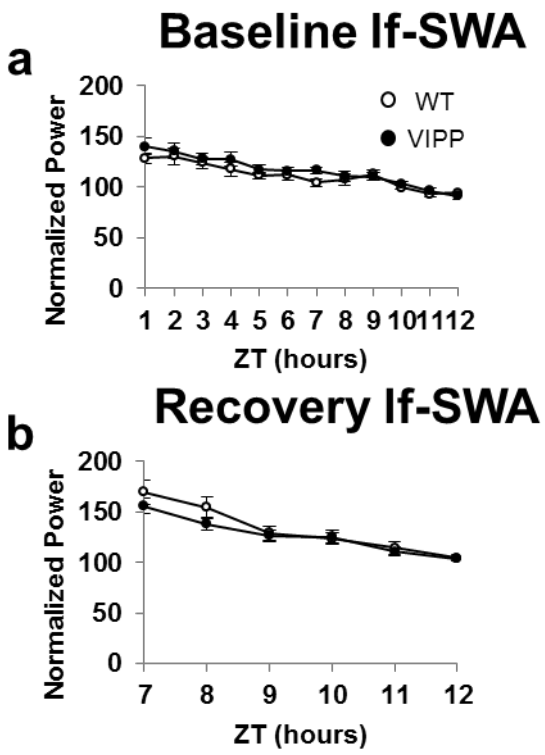


**Figure 11: VIPP mice exhibit normal SWA at baseline and after SD.** (A) Normalized lf-SWA in wild type and VIPP mice on the baseline day. VIPP and wild type mice had similar levels of lf-SWA on the baseline day from ZT0-12 (Two-way RM ANOVA; n.s.). (B) Normalized lf-SWA in wild type and VIPP mice after SD. VIPP and wild type mice had similar levels of lf-SWA after recovery from 6hr SD (Two-way RM ANOVA; n.s.).

dependent release in astrocytes. Attenuated $Ca^{2+}$ signaling in astrocytes did not recapitulate the dnSNARE phenotype. VIPP mice have normal lf-SWA power at baseline as well as after sleep deprivation (SD), compared to wild type mice (Figure 11), suggesting a unique role for astrocyte $IP_3/Ca^{2+}$ signaling in sleep regulation, separate from that of SNARE mediated release.

**Section 5.7: REM rebound sleep is enhanced when $IP_3/Ca^{2+}$ signaling is reduced in astrocytes.**

It is widely accepted that the homeostatic increase in SWS delta power that occurs with prolonged wake correlates with the duration of NREM sleep (McCarley 2007). REM sleep is also homeostatically regulated, with paradoxical sleep deprivation resulting in REM specific rebound sleep (Kitka, Katai et al. 2009); however the regulation of REM sleep homeostasis is poorly understood. Mice with $IP_3$ 5' phosphatase overexpressed in astrocytes show an increase in REM rebound sleep after six hours of sleep deprivation, compared to wild type (Figure 12). The greatest increase is seen in the later phase of the light cycle, during ZT10-11 (Figure 12c; $p<0.05$, Tukey's post hoc test). During the ZT6-12 time bin at baseline, VIPP mice have enhanced theta power at some frequencies during REM sleep ($p<0.05$ at 6.6-7.1Hz). However, when averaged across the entire theta range of 5-8Hz, VIPP mice show similar theta power to wild type (Figure 12e). VIPP mice show enhanced theta power during REM rebound sleep in the six hour period immediately following 6hr SD (Figure 12f; inset: wild type: 3.3±0.5; VIPP: 4.9±0.3). This suggests that the homeostatic increase in REM sleep is enhanced when $IP_3/Ca^{2+}$ signaling is reduced in astrocytes, indicating a regulatory role for astrocytic $IP_3/Ca^{2+}$ signaling in REM sleep and theta power.

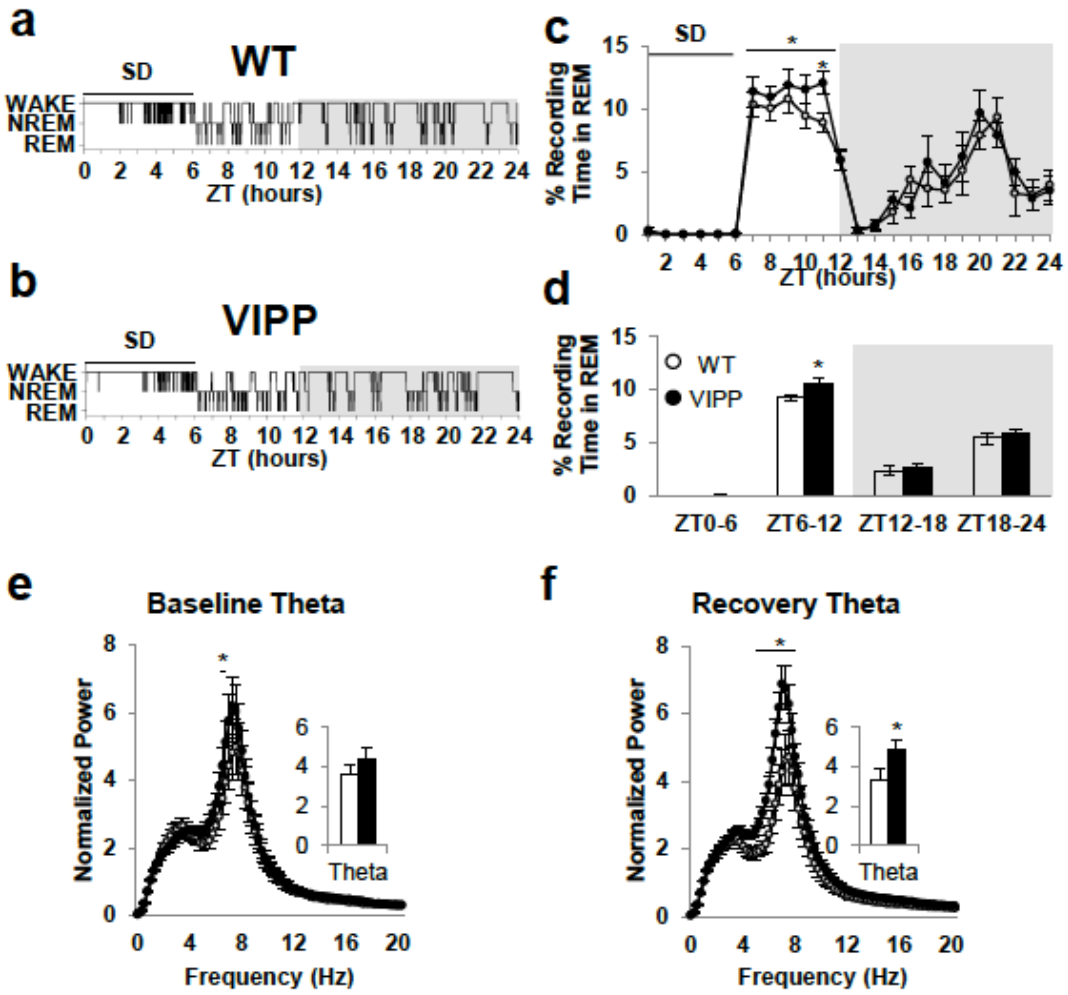


**Figure 12: Six hours of enforced wake enhances REM rebound in VIPP mice to a greater degree than wild type mice.** (A) Representative example of a wild type mouse hypnogram during (ZT0-6) and after SD (ZT6-24). (B) Representative example of a VIPP mouse hypnogram during (ZT0-6) and after SD (ZT6-24). (C) Percent time spent in REM sleep during the six hours of sleep deprivation and subsequent recovery period. VIPP mice spent significantly more time in REM sleep during the recovery period compared to wild type mice (ZT6-12: Two way RM ANOVA: *p<0.05; ZT11: Tukey's post hoc test: *p<0.05). (D) Average percent recording time spent in REM sleep. VIPP mice spend significantly more time in REM sleep after SD, compared to wild type mice (ZT6-12: t-test, *p<0.05). (E) Theta power at ZT6-12 during REM sleep on the baseline day. On the baseline day from ZT6-12 VIPP and wild type mice have enhanced theta power in select frequencies but similar average theta power (inset) (Two-way RM ANOVA: *p<0.05; inset: n.s.). (F) Theta power at ZT6-12 after SD. VIPP mice have significantly increased theta power from ZT6-12 after recovery from 6hr SD, compared to wild type mice (Two-way RM ANOVA: *p<0.05; inset: t-test, *p<0.05).

# Chapter 6: Discussion and Future Directions

## Section 6.1: Overview

Astrocytes have been recently promoted from being merely the "glue" that supports neurons, to key players in synaptic activity and homeostatic regulators of nervous system function. We now know they are integrally involved in neuronal activity and we are starting to learn more about how astrocyte function impacts behavior. Our results showing that a mouse model of attenuated gliotransmission exhibits a reduction in baseline mechanical nociception is the first indication that astrocytes modulate physiological pain behavior. We also show that gliotransmission contributes to baseline adenosine tone in the NAc, and in an absence of tonic adenosine activation on A1Rs, glutamate release is enhanced and post-synaptic MSN responses are increased. Finally, we show that a mouse model of attenuated $IP_3/Ca^{2+}$ signaling has a very different sleep phenotype from a mouse model of attenuated SNARE dependent gliotransmission, suggesting that discrete pathways of astrocyte signaling regulate different aspects of sleep. Furthermore, this was the first study to show that astrocyte $IP_3/Ca^{2+}$ signaling regulates REM sleep.

## Section 6.2: Astrocyte gliotransmission increases sensitivity to baseline mechanical nociception:

My results demonstrate that attenuated gliotransmission increases physiological baseline mechanical nociception. Given that administration of A1R agonists is known to decrease pain responses (Gong, Li et al. 2010) and that previous studies show dnSNARE mice exhibit reduced adenosine tone (Halassa, Florian et al. 2009), we speculate that the reduced baseline pain threshold measured in dnSNARE mice is due to decreased A1R activation in the spinal cord. This would suggest that an astrocytic source of adenosine

normally reduces acute pain signaling in the spinal cord. In the hippocampus, presynaptic A1R activation inhibits transmitter release and dnSNARE expression reduces tonic activation of these receptors (Schmitt, Sims et al. 2012). It may be that dnSNARE expression in the spinal cord leads to decreased tonic activation of presynaptic release sites on nociceptive neurons, thus allowing greater basal activity to persist and lowering pain threshold. Since AMPAR activation mediates baseline mechanical nociception, it is likely that dnSNARE mice have increased activation of these receptors due to a higher probability of release from nociceptive afferents. However, it should be stated that astrocytes in the cortex may differ from astrocytes in the spinal cord; thus, results of studies in cortex may not be directly applicable to those in the spinal cord. Furthermore, astrocytes are known to release a variety of chemical transmitters, and we cannot discount the possibility of additional contributions from other gliotransmitters such as D-serine. However, D-serine plays an excitatory role in the spinal cord, facilitating NMDAR activation, and mediating allodynia in NPP (Miraucourt, Peirs et al. 2011). Therefore, it is unlikely that attenuated release would cause increased pain by this mechanism.

As mentioned in the introduction to this topic, the spinal cord is not the only region in which pain signals are integrated. We examined the lumbar spinal cord for reactive astrocytes and microglia and did not find a difference between wild type and dnSNARE mice at baseline. We did not check supraspinal regions involved in pain signaling, such as the RVM or the ACC, so it is unknown whether there are baseline reactive astrocytes or microglia in one or both of these regions. The RVM facilitates descending nociceptive pathways and reactive glia in this region contribute to

neuropathic pain (Wei, Guo et al. 2008). Reactive astrocytes or microglia in this region as a result of dnSNARE expression may explain a decrease in baseline mechanical nociception, however this remains to be explored. The ACC is also a supraspinal brain region involved in regulation of pain perception. Many factors such as stress, depression, and sleep affect cortical function and astrocytes have been implicated in mediating these processes (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012, Hines, Schmitt et al. 2013, Hines and Haydon 2014). Importantly, increased glutamate release and AMPA receptor-mediated postsynaptic responses occur in the ACC after peripheral nerve injury (Xu, Wu et al. 2008). Considering that dnSNARE mice have decreased adenosine tone and increased postsynaptic responses in the hippocampus, it is possible that the ACC exhibits a similar phenotype. Increased glutamate transmission due to attenuated adenosine tone in the ACC is, thus, a possible mechanistic explanation for the decreased threshold to mechanical nociception seen in dnSNARE mice.

The model of attenuated gliotranmission via overexpression of a dominant negative peptide fragment of synaptobrevin II provides a global disruption in gliotransmission throughout the CNS. Given that dnSNARE mice have reduced NMDAR expression in the cortex, we hypothesized that the same would be true in the spinal cord. Though we did not directly test this, the fact that dnSNARE mice have reduced threshold to mechanical nociception disagrees with a reduction in NMDAR expression on dorsal horn neurons. A direct test for baseline effects of attenuated gliotransmission in the spinal cord would be to collect acute slices of living spinal cord and electrically evoked NMDAR function via patched dorsal horn neurons as well as NMDAR surface expression using biotinylation and Western Blotting for total protein levels. Biotinylation

of surface NMDAR expression compared to total NMDAR protein via Western Blotting will give a ratio of NMDA surface to total protein level that can be compared across genotypes.

Attenuated gliotransmission may impact multiple gliotransmitters. We did not directly test an effect of a specific gliotransmitter and could therefore only speculate on which was the likely candidate to increase baseline pain. Future studies to assess the specific gliotransmitter(s) that are decreased in the dorsal horn will elucidate mechanisms by which attenuated gliotransmission in dnSNARE mice increases baseline pain. Biosensors may be inserted into acute spinal cord slices to specifically assess D-serine or adenosine levels in wild type and dnSNARE mice. Furthermore, in vivo measurement of tonic adenosine in the dorsal horn may be achieved by recording field potentials in response to sciatic nerve stimulation.

Assessment of astrocyte and microglial reactivity in the RVM and ACC is necessary to rule out the possibility that the effect on baseline pain is due to supraspinal reactive gliosis. Yet another method to test where attenuated gliotransmission may mediate increased baseline mechanical nociception would be to locally transfect the lumbar spinal cord, the RVM, or the ACC and assess baseline pain behavior. Transduction of dnSNARE into astrocytes using AAV serotype 5 and dnSNARE driven by the GFAP promoter will allow for astrocyte specific expression that can be regionally controlled. Region-specific expression will provide information regarding which part of the pain pathway is sensitive to attenuated gliotransmission and results in increased baseline mechanical nociception.

**Section 6.3: Astrocyte gliotransmission contributes to baseline NAc adenosine tone; implications in cocaine-induced changes in plasticity**

Behavioral models of addiction as well as electrophysiological recordings from mice show excitatory transmission in the NAc and ventral tegmental area (VTA) are necessary for the development of cocaine-induced psychomotor sensitization (Kalivas and Alesdatter 1993, Wolf 1998, Wolf, Sun et al. 2004). Studies where GABA agonists or tetrodotoxin are infused into the PFC to block activity just before cocaine injection have shown that PFC afferents to the NAc are required for sensitization to cocaine and relapse to cocaine seeking behavior (Cornish and Kalivas 2000). Studies where AMPAR antagonists were infused into the NAc just prior to cocaine injection support that AMPAR activation is also required for behavioral sensitization to cocaine (McFarland and Kalivas 2001). Imaging studies have shown that PFC activation is diminished in withdrawn human addicts (Aron and Paulus 2007, Gu, Salmeron et al. 2010). This correlates with results from animal models that show decreased firing in PFC neurons, which synapse onto MSNs in the NAc. Additionally, extracellular glutamate levels are reduced by ~50% in the NAc of rodent models of addiction as a result of deactivation of the astrocytic cysteine-glutamate exchanger (Kalivas 2009). This reduces inhibition of pre-synaptic release governed by type II metabotropic glutamate receptors on synaptic terminals. Therefore, glutamatergic signaling is bidirectionally modified by means of decreased pre-synaptic firing and extracellular glutamate as well as reduced inhibition of release. In order to find new treatments for addiction, it is important to understand the baseline mechanisms that influence post synaptic MSNs in the NAc.

The primary goal of this study was to assess the role of astrocyte gliotransmission in baseline MSN function in the NAc. Previous studies in mice with attenuated gliotransmission revealed that intact astrocyte SNARE-mediated release of gliotransmitters is necessary for relapse behavior in conditioned place preference and self-administration in mouse models of addiction. Given that PFC and NAc activity are necessary for relapse behavior (McFarland and Kalivas 2001), and that mice with attenuated gliotransmission exhibit a failure to relapse to cocaine seeking (Turner, Ecke et al. 2013), we asked whether attenuated gliotransmission altered NAc physiology prior to cocaine exposure. We specifically isolated the PFC input to the NAc and asked whether gliotransmission affected signaling at this synapse. We assessed adenosine tone and glutamatergic function in the NAc, as they are both involved in mediating cocaine-induced changes in physiology that are correlated with relapse behavior.

Glutamate release from the PFC acting on AMPARs in the NAc is a crude requirement for cocaine seeking behavior in mice, but the pattern of glutamate signaling that is necessary for relapse behavior is unknown. Withdrawal from cocaine creates a deficit in glutamate signaling in the NAc that is thought to drive cocaine-seeking behavior (Kalivas and Hu 2006, Kalivas 2009). Simply decreasing basal AMPAR function in the NAc is sufficient to facilitate relapse (Bachtell and Self 2008). In the NAc, presynaptic A1R activation decreases glutamate release from PFC terminals (Ciruela, Casado et al. 2006). We found that in mice with attenuated gliotransmission, evoked glutamate release in the NAc is enhanced under baseline conditions. This was due to reduced adenosine tone acting on presynaptic A1Rs (Figure 2). Given these findings and that mice with attenuated gliotransmission fail to exhibit relapse behavior, one

explanation is that reduced A1R adenosine tone in the NAc of dnSNARE mice results in a dis-inhibition of glutamate release from PFC afferents, occluding the cocaine-induced increase in glutamate release that is required for relapse behavior. Another way in which reduced adenosine tone may inhibit relapse behavior stems from the hypothesis that in wild type mice, cocaine increases adenosine to levels that favor A2AR activation, which stimulates glutamate release in the NAc. In mice with attenuated gliotransmission, it is possible that adenosine levels never reach those required to activate A2ARs. Thus, the cocaine-induced increase in glutamate release at the PFC-NAc synapse does not occur; instead cocaine inhibits glutamate release by raising adenosine to levels that preferably activate A1Rs (from sub-A1R sensitivity levels). This may explain the slight aversion dnSNARE mice have when tested for CPP reinstatement and the lack of reinstatement of self-administration (Turner, Ecke et al. 2013).

Given the mounting evidence that adenosine signaling opposes dopamine signaling in the NAc (Azdad, Gall et al. 2009), one might speculate that mice with attenuated adenosine tone have increased responses to cocaine. However, this is not the case, suggesting a more complex explanation. In support of this, cocaine exposure increases adenosine tone in the VTA (Bonci and Williams 1996) and NAc (Fiorillo and Williams 2000), but decreases pre-synaptic adenosine receptor sensitivity (Manzoni, Pujalte et al. 1998) and decreases adenosine receptor expression (Marcellino, Roberts et al. 2007) in the NAc. Furthermore, the specific actions of adenosine depend on the heteromeric complexes the receptors form. A2ARs alone can form heterodimers with D2Rs, A1Rs, and mGluR5s (Ferre, Diamond et al. 2007). Studies showing D1R-mediated increases in cAMP argue the cocaine-induced increase in adenosine comes from neurons;

however it is possible that both astrocytic and neuronal sources of adenosine are necessary for relapse behavior. Since cocaine increases adenosine levels, perhaps to levels that favor A2AR activation in wild type mice (direct estimates of cocaine-induced elevations in adenosine are lacking), it is possible that mice with attenuated gliotransmission have very low adenosine levels at baseline, and cocaine brings adenosine to levels that preferably activate A1Rs and inhibit the cocaine-induced D1R activation and insertion of AMPARs that underlies cocaine craving and relapse behavior.

Findings by Deng et al. in the cortex suggest a postsynaptic signaling cascade mediated by astrocyte activation of A1Rs regulates NMDAR expression and function (Deng, Terunuma et al. 2011). However, A1Rs are highly expressed in the cortex and hippocampus, but are less expressed in the NAc. Given that the dnSNARE manipulation causes reduced pre-synaptic A1R adenosine tone in the NAc, but does not alter evoked NMDAR activity (Figure 5), it is possible that post synaptic A1R regulation of NMDAR expression is lower or less active in this brain region, compared to the hippocampus. Considering that A2ARs oppose A1R function and decreased gliotransmission likely results in decreased A2AR activation, there may be overall reduced A1R tone, but downstream signaling from A1R activation may be less inhibited in dnSNARE mice and result in no change in NMDAR function. Furthermore, since an underlying deficit in NMDAR expression doesn't seem to be the reason for the behavioral phenotype in dnSNARE mice, other explanations must be explored.

Although we only tested adenosine tone in the NAc of dnSNARE mice, evidence suggests astrocytes also release glutamate in a SNARE-dependent manner (Araque, Li et al. 2000, Bezzi, Gundersen et al. 2004). Furthermore, this astrocytic release of glutamate

affects synaptic strength in the hippocampus (Jourdain, Bergersen et al. 2007). Evidence also suggests astrocytes release D-serine in a SNARE-dependent mechanism (Mothet, Pollegioni et al. 2005). Considering its co-agonist properties, D-serine may modulate NMDAR mediated plasticity in the NAc. Indeed, D-serine levels are reduced in the NAc of cocaine treated rats (Curcio, Podda et al. 2013). However, the release of D-serine and glutamate have not been directly tested in the NAc of dnSNARE mice.

Plasticity in the NAc is indeed mediated by NMDAR-dependent changes in AMPARs, but this is not the only mechanism. LTP and LTD are also modulated by type I and type II mGluRs. Given the ability of astrocytes to modulate PFC-evoked glutamate release in the NAc, and that mGluR5 agonist application enhances $Ca^{2+}$-dependent glutamate release from astrocytes in the NAc (D'Ascenzo, Fellin et al. 2007), attenuated gliotransmission likely plays a role in mediating mGluR5 signaling, which is involved in cocaine-induced plasticity and reinstatement of cocaine seeking (Kumaresan, Yuan et al. 2009, Wang, Moussawi et al. 2013). As previously mentioned, an astrocytic source of adenosine indirectly regulates presynaptic release of glutamate in the NAc. If this source of glutamate were affecting mGluR activation due to increased spillover as a consequence of disinhibited release, the effects on mGluRs would be reversed. In reality, it is likely that both synaptic and extrasynaptic levels of glutamate are altered in dnSNARE mice. Future studies should be directed at determining the role of gliotransmission in glutamate levels in the NAc at baseline and after cocaine exposure.

The gliotransmitters adenosine and glutamate have been shown to influence spontaneous and evoked EPSCs through activation of presynaptic A1Rs and mGluRs in the hippocampus (Martin, Fernandez et al. 2007, Perea and Araque 2007). Furthermore,

withdrawal from cocaine increases mEPSC frequency and amplitude, and these changes mediate relapse. We did not see an effect of attenuated gliotransmission on baseline mEPSC frequency (Figure 4). Given that dnSNARE mice have reduced pre-synaptic A1R tone, which decreases evoked release in the NAc (Figure 2), a lack of an effect on action potential-independent release indicates that astrocyte-dependent A1R activation does not regulate spontaneous glutamate release in the NAc.

ATP and adenosine signaling activate $K^+$ channels, which hyperpolarize the resting membrane potential of neurons (Isomoto, Kondo et al. 1997). In spite of this, we found that MSNs in the NAc of dnSNARE mice had unaltered resting membrane potentials compared to wild type mice (Figure 3). This suggests an astrocytic source of tonic adenosine does not regulate the activity of $K^+$ channel conductance relevant to MSN resting membrane potential. Conversely, MSNs from dnSNARE mice did exhibit increased excitability, characterized by increased action potential number at lower current injections. These data indicate astrocyte gliotransmission regulates MSN action potential firing, a function that has not previously been demonstrated. In support of a role for astrocyte-mediated gliotransmitter release in dampening neuronal spiking, Newman has shown that glial activation in the retina causes decreased neuronal spike frequency in an A1R-dependent manner (Newman 2003).

For this investigation, we focused primarily on the PFC to NAc synapse. However, glutamatergic afferents from the amygdala also mediate cue-induced drug seeking (Di Ciano and Everitt 2001). Furthermore, gliotransmission could alter synaptic transmission upstream of the NAc by affecting input or excitability of PFC neurons, ultimately affecting their output to the NAc. The VTA, which is involved in reward-

related learning and release of dopamine to modify glutamate signaling in the PFC and NAc, may also be affected by attenuated gliotransmission. However, an effect of attenuated gliotransmission on cocaine-induced changes in the VTA would likely surface before the point of relapse because single cocaine injections change the physiology of this synapse (Bowers, Chen et al. 2010). Nevertheless, isolation of specific synapses via optogenetics or precisely placed stimulating and recording electrodes would reveal whether altered signaling at another synapse is what underlies the deficit in relapse behavior exhibited by dnSNARE mice.

In summary, we focused on the effects of attenuated gliotransmission on basal NAc physiology. We found a reduction in adenosine tone and a corresponding increase in evoked presynaptic release of glutamate. We did not see an effect of attenuated gliotransmission on post-synaptic glutamate receptor function. Considering the importance for AMPA and NMDARs in mediating cocaine-induced changes in NAc plasticity, the function of these receptors should be tested in dnSNARE mice during cocaine exposure and after withdrawal from chronic cocaine. Since dnSNARE mice are able to learn to self-administer cocaine and develop addictive lever pressing behavior, similar to wild type mice, but fail to relapse after extinction training, it is possible that attenuated gliotransmission prevents cocaine-induced changes in plasticity at a specific point in cocaine exposure. Cocaine activated brain regions during relapse can be determined by measuring c-fos immunoreactivity in dnSNARE and wild type mice. A baseline deficit in adenosine might prevent the changes in NAc activation and plasticity that are associated with incubation of cocaine craving and increased propensity to relapse.

**Section 6.4: Astrocyte IP3/Ca2+ signaling regulates theta rhythms and REM sleep, while SNARE-dependent release from astrocytes regulates NREM and sleep homeostasis**

Sleep is essential to all species and loss of sleep can be detrimental to the health and well-being of animals and humans. However, the regulation of sleep is poorly understood. Our lab has previously shown a role for astrocyte gliotransmission in regulating the homeostatic drive to sleep (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012). For this study, we sought to understand the role astrocytic $IP_3/Ca^{2+}$ plays in sleep regulation.

REM sleep is dominated by neuronal activity in the theta frequency band, which is strongest in the hippocampus (Everitt and Robbins 1997). Theta activity and cholinergic activity in the hippocampus are tightly coupled (Zhang, Lin et al. 2010). Astrocytes express receptors for and are activated by ACh. Indeed, hippocampal astrocytes respond to activity-dependent release of ACh by elevating their intracellular $Ca^{2+}$ (Araque, Martin et al. 2002). As previously mentioned, $Ca^{2+}$ elevations in astrocytes result in a plethora of downstream activations, including gliotransmission and neuronal receptor activation (Kreft, Stenovec et al. 2004). We found that VIPP mice have enhanced theta power, particularly during REM sleep (Figure 10), suggesting a role for astrocyte $IP_3/Ca^{2+}$ signaling in regulating hippocampal theta power. One way this may occur is through cholinergic activation of astrocytes. Indeed, cholinergic-induced plasticity in the hippocampus requires astrocyte $Ca^{2+}$ elevations (Navarrete, Perea et al. 2012). The complexity of cholinergic control of the hippocampus is vast, and our understanding of how cholinergic input regulates excitation, inhibition, and astrocyte

function is incomplete (McQuiston 2014). Given that astrocytes respond to cholinergic input and mediate cholinergic signaling in the hippocampus, and that mice with attenuated $IP_3/Ca^{2+}$ signaling in astrocytes have enhanced theta power, astrocytes may be a new target for investigation of theta regulation, a poorly understood phenomenon.

Increased theta power is correlated with an increase in REM sleep in VIPP mice. Mice with attenuated $IP_3/Ca^{2+}$ signaling specifically in astrocytes spent more time in REM sleep than wild type mice (Figure 8). This was likely due to the fact that VIPP mice entered REM sleep more frequently (Figure 7d). Mice with attenuated $IP_3/Ca^{2+}$ signaling may enter REM sleep more frequently because they lack the ability to initiate a $Ca^{2+}$-dependent inhibition of REM sleep onset. The increase in REM bouts is small, but significant. An interesting parallel to this phenotype is that depressed humans have slight increases in REM sleep time.

Mood disorders are associated with increased cholinergic to aminergic neurotransmitter levels (Costa e Silva 2006). Accordingly, increased REM density, characterized by increased frequency of rapid eye movements, is an endophenotype of depression (Steiger and Kimura 2010). Increased REM sleep rebound after SD is also present in models of depression (Steiger and Kimura 2010). Treatment with antidepressants decreases time spent in REM and discontinuation of medication causes rebound REM sleep. Previous investigation has shown that astrocytes are involved in mediating the antidepressant effects of sleep deprivation, a commonly used but poorly understood method of acute treatment for major depression (Hines, Schmitt et al. 2013). Given that astrocytes respond to cholinergic and aminergic stimulation by elevating intracellular $Ca^{2+}$ concentration (Costa e Silva 2006, Ding, O'Donnell et al. 2013,

Paukert, Agarwal et al. 2014), and mice with attenuated astrocytic $Ca^{2+}$ signaling show increased REM sleep before and after SD, compared to wild type mice, this mechanism may provide insight into the link between depression and REM sleep: furthermore, astrocytes may serve as a new therapeutic target for treatment of psychiatric diseases and sleep disorders.

The homeostatic drive to sleep is characterized by increased slow wave activity delta power, or sleep pressure (McCarley 2007). Accumulation of an astrocytic source of adenosine increases sleep drive (Halassa, Florian et al. 2009, Schmitt, Sims et al. 2012). REM sleep is also homeostatically regulated, with a clear increase in percent time spent in REM after paradoxical sleep deprivation (Kitka, Katai et al. 2009), but the mechanism by which this occurs is largely unknown. Interestingly, mice with attenuated $IP_3/Ca^{2+}$ signaling in astrocytes showed increased REM sleep rebound after six hours of sleep deprivation, compared to wild type (Figure 12), but exhibited a sleep deprivation induced increase in delta power similar to wild type (Figure 11). These data suggest that there are distinct astrocytic processes that regulate different aspects of sleep and cortical function in general. In fact, a recent study showed tetanus toxin disruption of vesicular mediated release from astrocytes decreased gamma (25-80Hz) frequency activity (Lee, Ghetti et al. 2014). Taken together, these studies demonstrate a previously unrecognized complexity of astrocytic modulation of neuronal networks and cortical rhythm generation.

Given that previous studies disrupting vesicular-mediated release from astrocytes revealed a role for astrocyte gliotransmission in regulating NREM but not REM sleep, and we found that disrupting $IP_3/Ca^{2+}$ signaling affected REM but not NREM sleep, the pathways by which astrocytes regulate REM and NREM sleep must be non-overlapping.

In the dnSNARE manipulation of gliotransmission, a dominant negative portion of Sb2 was expressed. Sb2-containing SNARE complexes are involved in $Ca^{2+}$-dependent vesicular release (Pascual, Casper et al. 2005, Liu, Sun et al. 2011). A distinct phenotype from what we see when we disrupt $IP_3$-dependent $Ca^{2+}$ signaling, the most widely studied mechanism of calcium signaling, suggests another source of $Ca^{2+}$ activates SNARE-mediated release. Indeed, other previously undetectable sources of $Ca^{2+}$ signaling have been discovered using a new technology where genetically encoded membrane tethered $Ca^{2+}$ indicators can detect microdomains of $Ca^{2+}$ influx from the extracellular space. One source of this extracellular $Ca^{2+}$ influx is dependent on TRPA1 receptors (Shigetomi, Bushong et al. 2013, Shigetomi, Jackson-Weaver et al. 2013, Tong, Shigetomi et al. 2013, Jiang, Haustein et al. 2014). This microdomain signal would not be detectable with the Rhod2 indicator dye, which was used to measure large cell-wide evoked $Ca^{2+}$ signals to confirm the dramatic reduction in $Ca^{2+}$ signal seen in VIPP mice (Schmitt et al., unpublished). The results obtained from this study suggest astrocyte $IP_3/Ca^{2+}$ signaling is not necessary for SNARE-mediated gliotransmission that regulates NREM delta power and SWS homeostasis. Furthermore, the mechanism by which astrocyte $IP_3/Ca^{2+}$ signaling modulates REM sleep and theta power is likely to be independent of SNARE-mediated gliotransmission, as previous work has shown these measures are unchanged in dnSNARE mice (Halassa, Florian et al. 2009).

Considering that NREM sleep and delta power are intact in VIPP mice, but are attenuated in dnSNARE mice, this suggests the $Ca^{2+}$-dependent mechanism for SNARE-mediated release that likely affects NREM, is intact when $IP_3$ signaling is disrupted. Moreover, it is likely that $IP_3/Ca^{2+}$ signaling in astrocytes targets another mechanism that

regulates REM sleep via release of gliotransmitters through a non-SNARE dependent mechanism. Alternatively $IP_3/Ca^{2+}$ signaling may regulate REM sleep through a mechanism other than gliotransmission such as glutamate uptake, which is regulated by activity dependent activation of mGluR1 and $IP_3/Ca^{2+}$ (Devaraju, Sun et al. 2013). Another explanation is that activity-dependent activation of mGluRs on astrocytes elevates $IP_3/Ca^{2+}$ and regulates synaptic coverage by PAPs (Bernardinelli, Randall et al. 2014). The proximity of the PAP to the synapse governs the extent of glutamate uptake by GLT-1. Given that VIPP mice have attenuated $IP_3/Ca^{2+}$ signaling, structural plasticity of the astrocyte and synaptic coverage may be reduced in VIPP mice, allowing for a hyper excited synapse. Recent evidence indicates that astrocyte GLT-1 expression regulates excitability in the lateral habenula (Cui, Mizukami et al. 2014).

The lateral habenula has recently been implicated in REM sleep regulation. The output of this glutamatergic synapse regulates the firing of monoaminergic neurons in the brain stem (Aizawa, Cui et al. 2013, Luo, Zhang et al. 2014). Additionally, lateral habenula neurons fire in synchrony with hippocampal theta during REM sleep (Aizawa, Cui et al. 2013, Goutagny, Loureiro et al. 2013). Hyperexcitability of this synapse results in increased REM sleep. Blocking astrocytic GLT-1-dependent glutamate uptake specifically in the lateral habenula increased postsynaptic neuron firing rate and increased REM sleep (Cui, Mizukami et al. 2014). Knocking out GLT-1 in the lateral habenula also caused a depressive phenotype in mice. In VIPP mice, β-Gal reporter expression is particularly high in the lateral habenula. Therefore, astrocyte $IP_3/Ca^{2+}$ signaling may regulate theta rhythms and REM sleep by mediating group I mGluR-induced $Ca^{2+}$ increases to modulate glutamate uptake, either directly through transporter expression, or

indirectly through synaptic coverage. Also, SNARE-mediated release from astrocytes may not involve $IP_3$-dependent $Ca^{2+}$ release, but a small source of localized $Ca^{2+}$ that enters from the extracellular space. Morphological evidence of astrocyte coverage in the lateral habenula, in combination with immunohistochemical measurement of GLT-1 expression levels in VIPP expressing versus wild type astrocytes, will provide insight to the glutamate transport capabilities in VIPP expressing astrocytes.

Future studies to determine mGluR1 activation of $IP_3$/Ca2+ signaling in astrocytes and the ability of VIPP expressing astrocytes to respond to mGluR1 activation and regulate GLT-1 expression in the lateral habenula may reveal a direct link between astrocyte $IP_3/Ca^{2+}$ signaling and REM sleep. Furthermore, given that hyperexcitability of the lateral habenula results in a depressive phenotype in a social defeat paradigm, this same test should be run in VIPP mice. As the gold standard for testing a mouse for depression is determining whether antidepressant treatment reverses the depressed phenotype, administration of antidepressant drugs after the social defeat paradigm should be tested for reversal of the depressed phenotype in VIPP mice. Finally, testing whether antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), decrease REM sleep time in VIPP mice (as they do in humans) will determine whether astrocyte $IP_3/Ca^{2+}$ signaling is involved in regulating REM sleep and depression.

Gliotransmission and astrocyte $Ca^{2+}$ signaling affect neuronal activity in many ways (Araque, Carmignoto et al. 2014). The present study examined the effect of attenuated $IP_3$-dependent $Ca^{2+}$ signaling in astrocytes and found that they play a modulatory role in REM sleep, but did not recapitulate the sleep phenotype seen in mice with attenuated gliotransmission, which was attenuated NREM sleep homeostasis. Given

this finding, VIPP and dnSNARE genetic manipulations may target discrete molecular pathways, different subpopulations of astrocytes, and/or express at higher levels in different brain regions. A way to test the hypothesis that the two transgenes affect different brain regions would be to quantify transgene expression across brain regions in each genotype. If brain region expression is comparable across genotypes, crossing dnSNARE and VIPP mice and testing for an additive effect of their sleep phenotypes will confirm that VIPP and dnSNARE target different sub-populations of astrocytes or discrete molecular signaling pathways within astrocytes.

A very recent study made use of a Gq-coupled protease activated receptor 1 (PAR1) agonist in VIPP mice to demonstrate that astrocytic $IP_3/Ca^{2+}$ signaling mediates release of adenosine from hippocampal astrocytes (Schmitt et al., unpublished). This experiment was repeated in dnSNARE mice to confirm that both $IP_3/Ca^{2+}$- and SNARE-mediated release were necessary for PAR1-evoked increases in extracellular adenosine. This study also highlighted the role for $IP_3/Ca^{2+}$ signaling in enhancing NMDAR signaling in the hippocampus as PAR1 agonist application increased NMDAR fEPSPs in wild type, but not VIPP mice. Furthermore, it was confirmed that the increase in NMDAR function in wild type mice was dependent upon A1R activation, as it was attenuated by an A1R antagonist. These data suggest that adenosine mediated release in the hippocampus is dependent on $IP_3/Ca^{2+}$ signaling after all. A final explanation for how VIPP and dnSNARE mice could have completely opposite sleep phenotypes is that dnSNARE expression is higher in the basal forebrain, where adenosine accumulation inactivates wake-promoting neurons. Expression in this region may have a higher effect on NREM, where VIPP is highly expressed in the lateral habenula, possibly favoring an

effect on REM sleep. Furthermore, it cannot be ruled out that VIPP and dnSNARE are somehow affecting two distinct populations of astrocytes that play different roles in regulating sleep in vivo that is not obvious when measured in hippocampal slices. Future studies should be directed at determining region specific effects of VIPP and dnSNARE transgene expression via virus-induced expression. These studies will likely reveal specific roles for each signaling pathway in astrocytes, which may overlap in some regions, but be mutually exclusive in others.

Although the mechanism remains unclear, this is the first study to show that *in vivo* manipulation of astrocyte $Ca^{2+}$ signaling directly affects behavior. Furthermore, this study revealed a unique regulatory mechanism of astrocyte signaling in sleep regulation, independent of SNARE-mediated gliotransmission, which contributes to REM, but not NREM sleep. Since increased REM sleep is an endophenotype for depression, understanding the pathway by which astrocyte $Ca^{2+}$ signaling regulates theta rhythm and REM sleep may elucidate targets for treatment of sleep-related and psychiatric disorders.

**Section 6.5: Overall discussion**

The study of astrocytes has come a long way in the past two decades, but is still in its infancy. The sections in this discussion provide a strong argument for a role for gliotransmission and astrocyte $IP_3/Ca^{2+}$ signaling in different aspects of mouse behavior, but the signaling cascades beyond gliotransmitter release remain elusive. Future studies should be aimed at understanding not only whether gliotransmission influences behavior, but also specifically what gliotransmitters are released in which brain region and how they affect behavior. Other studies that will provide insight into how astrocytes regulate

brain function will address whether gliotransmitters are co-released, which gliotransmitters are released in a SNARE-dependent mechanism under what circumstances, which processes are $Ca^{2+}$-dependent, and specifically how the $Ca^{2+}$ signal is encoded spatially and temporally.

In summary, the central nervous system is a massive web of interconnected neurons and glial cells that modulate connectivity in ever changing ways, adapting to the environment. Slight alterations of neuron or glial function result in diseased states of the brain. Yet, even at sub-optimal function, the brain is capable of immense processing power. We have yet to fully understand this system that enables us to think and try to understand it, but integrating the function of a new cell type, the astrocyte, which was previously thought not to be involved in information processing may give us better hope of some day figuring it out.

> "If our brains were simple enough for us to understand them, we'd be so simple that we couldn't"
>
> -Ian Stewart

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