Control of hippocampal synaptic transmission and plasticity by wakefulness-dependent elevation of astrocyte-derived adenosine

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Abbreviations:

aCSF: artificial Cerebro-Spinal Fluid

ADO: Amperometric Adenosine Biosensor

adorA1R: Adenosine A1 Receptor

ADP: Adenosine di-Phosphate

AMP: Adenosine mono-Phosphate

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

ATP: Adenosine tri-Phosphate

BF: Basal Forebrain

CA1: Cornu Ammonis Area 1

cAMP: cyclic-Adenosine mono-Phosphate

CNS: Central Nervous System

CPT: 8-Cyclopentyl-1,3-dimethylxanthine

dnSNARE: Transgenic Mouse Model Expressing dominant Negative SNARE specifically in Astrocytes

DPCPX: 8-Cyclopentyl-1,3-dipropylxanthine

EEG: electroencephalographic

EMG: electromyographic

ENT1: Equilibrative Nucleotide Transporter 1

ER: Endoplasmic Reticulum

fEPSP: field Excitatory Post-Synaptic Potential

FFT: Fast Fourier Transform

GECI: Genetically Encoded Calcium Indicator

GFAP: glial fibrillary acidic protein

GPCR: G-protein Coupled Receptor

i.c.v.: intracerebroventricular

IMP: Inosine mono-Phosphate

INO: Amperometric Inosine Biosensor

InsP3Rs: IP_3 receptors

IODO: Iodotubericidin

i.p.: Intra Peritoneal

IP_3: Inositol tri-phosphate

LDT: Laterodorsal Tegmental Nucleus

LFP: Local Field potential

LTP: Long Term Potentiation

mEPSC: Miniature Excitatory Post-Synaptic Current

mGluR: Metabotropic Glutamate Recepotr

MTEP: 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine

NBMPR: 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine

NMDA: N-methyl-D-aspartate

NMDAR-fEPSPs: NMDA receptor mediated fEPSP

NREM: Non-REM Sleep

PCR: Polymerase Chain Reaction

PKA: Protein Kinase A

PLC: Phospholipase C

REM: Rapid Eye Movement Sleep

SIC: Slow Inward Current

SWA: Slow Wave Activity (0.5-4 Hz)

tTA: tetracycline trans-activator

VAMP2: Vesicle Associated Membrane Protein 2

VIPP: Venus-tagged IP_3 Phosphatase

W: Wakefulness

WT: Wild Type or single transgene control

Abstract

Sleep is a highly conserved behavior which is critically important to the function of the nervous system and in particular to consolidation of declarative memory. Recent evidence has suggested that astrocytes, the major glial support cell within the brain, play a key role in maintaining sleep homeostasis by governing the level of adenosine which acts in extracellular space to regulate synaptic transmission. In addition, adenosine signaling by these cells has been implicated in the regulation of hippocampal dependent memory, suggesting that they play a previously unrecognized role in information processing within this brain area. Although it is increasingly evident that astrocytes can listen and respond to neurotransmission through calcium dependent exocytotic release, the mechanism by which they respond to wakefulness to affect synaptic transmission remains poorly understood.

In this dissertation, I present the results of my investigations into the role of astrocyte-derived adenosine in wakefulness-dependent changes to synaptic transmission and neuroplasticity within the hippocampus. Using a variety of electrophysiological means combined with molecular genetic disruption of astrocytic exocytosis and Inositol tri-Phosphate (IP_3) signaling in these cells, I demonstrate for the first time that wakefulness can regulate the level of astrocyte-derived adenosine acting in the hippocampus. I show that acute loss of sleep leads to an increase in signaling by this transmitter, while extended sleep loss produces an allostatic adjustment to reduce the level of adenosine, probably through reduced release, thereby compensating for the effects of chronic sleep disruption. Finally, I show that the mechanism by which astrocytes control extracellular adenosine depends on IP_3 calcium mobilization in these

cells and that over short time scales astrocyte-derived adenosine can both inhibit synaptic release of glutamate and enhance synaptic plasticity by increasing NMDA receptor mediated responses. Because of the critical importance of sleep in maintaining the health of the nervous system and the comorbidity of sleep pathologies in various neurological and psychiatric disorders, these results hold important implications for understanding the role of both astrocytes and adenosine signaling in health and disease.

Chapter 1: Background and Significance

Section 1.1: Introduction

Astrocytes, once considered primarily a passive support cell type responsible for meeting metabolic demands of neurons and maintaining potassium homeostasis, are now known to respond to and regulate neurotransmission to influence information processing and behavior. Although astrocytes are not electrically excitable, they express a variety of receptors that when activated, trigger different signaling cascades to control cytosolic calcium, primarily by mobilizing it from intracellular stores including the endoplasmic reticulum (ER) and mitochondria (Berridge, 2006; Pivneva et al., 2008; Zorec et al., 2012). This type of calcium response then leads to release of signaling molecules including amino acid (such as d-serine and glutamate), purine (such as adenosine tri-phosphate: ATP), and peptide transmitters (such as brain derived neurotrophic factor: BDNF); a phenomenon termed gliotransmission (Zhang and Haydon, 2005; Halassa et al., 2007a; Parpura and Zorec, 2010). Because astrocytic processes envelop synapses and dendritic spines as well as neuronal somata (Reichenbach et al., 2010) the release of these 'gliotransmitters' can act on both the presynaptic terminal and postsynaptically on the

dendrite to modulate synaptic transmission (Arrigoni et al., 2001; Fellin et al., 2004; Perea and Araque, 2007). Although the territory of a single astrocyte is relatively restricted, their finely divided processes allow them to act locally on large numbers of dendritic arbors. Indeed, the territory of a single hippocampal astrocyte has been shown to include 300-600 dendrites and with astrocytic filaments contacting approximately 100,000 synapses (Oberheim et al., 2006; Halassa et al., 2007b; Reichenbach et al., 2010). This complex and highly specialized morphology suggests that these cells may play a key role in regulating neuronal network dynamics. Consistent with this idea, recent studies conducted in the Haydon laboratory and others have demonstrated that astrocytes synchronize network activity in both the hippocampus (Angulo et al., 2004; Fellin et al., 2004) and cortex (Crunelli et al., 2002; Fellin et al., 2009). To date, however, the relevance of this type of astrocytic control to synaptic and network function is imperfectly understood.

Changes in astrocytic morphology and astrocyte specific gene expression are present in a variety of neurological and psychiatric disorders (Wulff et al., 2010); suggesting that astrocytes may participate in the initiation or progression of neurological disease states. This association, coupled with the capacity of astrocytes to regulate synaptic transmission *in situ*, strongly implies a role for astrocytes in higher order brain function. However, the direct involvement of astrocytic release in behavior has only recently been demonstrated. Using a transgenic mouse model in which vesicular release from astrocytes is disrupted by a dominant negative transgene, the dnSNARE mouse, Halassa and others showed that astrocytes control the homeostatic sleep response (Halassa et al., 2009b). Disrupting vesicular release from these cells prevented the

increase in sleep following sleep deprivation and reduced other markers of sleep drive, including low frequency, slow wave, electroencephalographic (EEG) activity (Fellin et al., 2009; Halassa et al., 2009b). Several lines of evidence suggest that this form of sleep homeostasis is mediated by increased activation of adenosine A1 receptors (adorA1Rs) (Alam et al., 1999; Basheer et al., 2004; Blutstein and Haydon, 2013). Because astrocytes are known to release ATP (Guthrie et al., 1999; Pangrsic et al., 2007), which is rapidly converted to adenosine in the extracellular space (Dunwiddie et al., 1997), this finding implies that astrocytes are responsible for wakefulness-dependent changes in adenosine.

In addition to its importantce in maintaining sleep homeostasis, further investigation using the dnSNARE mouse also demonstrated that astrocytic release was involved in the disruption of hippocampal dependent spatial memory observed following sleep deprivation. Blocking adorA1 receptors similarly rescued memory consolidation after sleep loss suggesting that the effect of sleep deprivation involves signaling by adenosine (Halassa et al., 2009b; Florian et al., 2011). These findings imply that astrocyte-derived adenosine plays a dynamic role in regulating hippocampal function in response to wakefulness. Previous studies using microdialysis have reported wakefulness-dependent elevation of adenosine in areas not directly associated with wakefulness, including the cortex as well as the basal forebrain (Christie et al., 2008; Kalinchuk et al., 2011). In the hippocampus, however, both the regulation of astrocyte-derived adenosine by wakefulness and its impact on synaptic function and plasticity remain unexplored.

The research presented in this dissertation was designed to determine how wakefulness affects astrocyte-derived-adenosine and to investigate the effects of calcium-dependent elevation of this gliotransmitter on synaptic function and plasticity. To accomplish this, I have applied electrophysiological and electrochemical methods to investigate the effect of perturbing gliotransmission and astrocytic calcium signaling. To disrupt these processes I have employed conditional, astrocyte specific transgene expression. Specifically, I have used these approaches to 1) test the hypothesis that wakefulness increases astrocyte-derived adenosine in the hippocampus and cortex and that this increase depends on vesicular SNAP (Soluble NSF Attachment Protein) Receptors (SNAREs); 2) determine the effect of protracted sleep interruption on wakefulness-dependent control of extracellular adenosine; and 3) assess whether IP_3 mediated astrocytic calcium elevation can increase the concentration of astrocyte-derived adenosine to produce metaplastic changes in neuronal NMDA receptor (NMDAR) function, thereby lowering the threshold for long term potentiation (LTP).

In order to frame this work I will first introduce the subject matter by describing the background and rationale for the research presented. In the second chapter I will explain the techniques applied in order to establish the benefits and limits of each approach. The following chapters will then describe the results obtained in the course of my research. Finally, I will discuss the conclusions which can be drawn from these findings and the implications for the involvement of astrocytes in normal behavior and

disease states, with special emphasis on the involvement of these cells in sleep-associated pathologies.

Section 1.2: Astrocytes Release Transmitters through Calcium Dependent Exocytosis

The discovery that electrically silent astrocytes are capable of sensing and responding to synaptic activity has begun a revolution in scientific understanding of the involvement of these cells both in information processing and in diseases of the nervous system (Araque et al., 1999; Haydon, 2001; Fellin et al., 2006; Parpura et al., 2010; Zorec et al., 2012; Blutstein and Haydon, 2013). Central to this process is the capacity of astrocytes to release gliotransmitters though calcium dependent mechanisms (Araque et al., 2000; Parpura et al., 2011; Navarrete et al., 2013). Astrocytic calcium signaling is mediated by multiple mechanisms including entry from the extracellular space and mobilization from intracellular stores including mitochondria and the endoplasmic reticulum (ER) (Zorec et al., 2012). The tight coupling between calcium permeable channels and vesicular release machinery observed for voltage gated channels in the presynaptic terminals of neurons does not appear to be present in astrocytes, however, suggesting that a higher and more widely distributed elevation in cytosolic calcium may be required for vesicular fusion in these cells (Hamilton and Attwell, 2010).

Liberation of calcium from intracellular organelles can provide large extended elevations in cytosolic calcium through multiple mechanisms (Hajnóczky and Thomas, 1997; Arcuino et al., 2002). This type of calcium elevation is generally triggered by signaling cascades activated by G-Protein Coupled Receptors (GPCRs) including

purinergic P_{2y} receptors and metabotropic glutamate receptors (mGluRs)(Shelton and Mccarthy, 1999; Fellin et al., 2006; Devaraju et al., 2013). A variety of receptors are expressed in astrocytes throughout the CNS allowing astrocytes to respond to a diverse array of signals. Indeed, individual astrocytes within the hippocampus may display a calcium response to activation of at least four different receptors (Shelton and McCarthy, 2000). Many of these receptors are associated with Gq alpha subunits and, once activated, signal through Phospholipase C (PLC) to generate Inositol tri-phosphate (IP_3). This activates IP_3 receptors (InsP3Rs) which are present on ER membranes (Neves et al., 2002; Billups et al., 2008). Once activated, InsP3Rs permit calcium release from the endoplastic reticulum into the cytosol (Hajnóczky and Thomas, 1997) where it can couple to various calcium dependent signaling pathways or cause transmitter release through SNARE dependent vesicular fusion (Araque et al., 2000) and other mechanisms (Lee et al., 2010; Liu et al., 2011; Han et al., 2013).

A canonical example of this type of calcium dependent response is observed following activation of metabotropic, Gq coupled mGluR1/5 receptors. In astrocyte/neuron co-cultures, these receptors have been shown to allow astrocytes to respond reciprocally to glutamate released from excitatory pyramidal neurons by releasing glutamate themselves via SNARE dependent vesicular exocytosis (Araque et al., 2000). This astrocyte-derived glutamate can propagate the calcium signal generated by neurons to other astrocytes (Cornell-Bell et al., 1990) and is also thought to be responsible for slow inward currents (SICs) observed in patch clamp recordings of neurons. These have been shown to occur following astrocytic calcium events in cultured astrocytes and *in situ* (Araque et al., 2000; Angulo et al., 2004; Fellin et al., 2004). Such

prominent inward currents are thought to be produced by glutamate released from the astrocyte acting on extra-synaptic NMDA receptors and can lead to activation of a variety of downstream signaling pathways (Perea and Araque, 2010). Although astrocytic glutamate release has been observed by multiple groups *in situ* its existence and functional impact under physiological conditions *in vivo* remains controversial (Hamilton and Attwell, 2010).

Considerable evidence suggests that astrocytic InsP3R mediated calcium responses occur under various conditions and that they are sufficient to produce vesicular and non-vesicular calcium dependent release (Haydon, 2001; Parpura et al., 2010, 2011; Zorec et al., 2012). Despite this evidence, however the precise function of this form of calcium response remains controversial (Agulhon et al., 2008; Hamilton and Attwell, 2010). This uncertainty arises in part from discrepancies in the observations made in studies attempting to determine the nature of astrocytic calcium signaling *in vivo* (Rusakov et al., 2011). While several studies have identified both spontaneous (Hirase et al., 2004; Takata and Hirase, 2008; Kuga et al., 2011) and induced (Takata et al., 2011; Thrane et al., 2012) calcium activity in astrocytes within the intact mouse cortex there remains ongoing debate as to whether global calcium activity is physiologically relevant in astrocytes associated with synapses (Shigetomi et al., 2013a) or whether large scale calcium elevations are predominantly associated with vascular regulation (Winship et al., 2007). This controversy is partly due to the differences in observed calcium dynamics between studies. Depending on the intensity and type of stimulations, astrocytic calcium elevations in some studies appear as large waves propagating among multiple astrocytes (Kuga et al., 2011) while in others activity is highly localized (Bernardinelli et al., 2011).

This apparent discrepancy may be due to differences in the experimental method since buffering by calcium sensitive dyes can abrogate spontaneous and evoked calcium dynamics (Henneberger and Rusakov, 2010; Rusakov et al., 2011). This interpretation is supported by recent experiments employing surface targeted Genetically Encoded Calcium Indicators (GECIs) with minimal buffering capacities in which highly localized spontaneous calcium signaling events were frequently observed in the majority of astrocytes expressing these calcium indicators (Rusakov et al., 2011; Shigetomi et al., 2013a, 2013b). In addition, several studies have shown that sensory stimulation is sufficient to drive astrocytic calcium (Hirase et al., 2004; Winship et al., 2007), demonstrating that physiological stimuli can induce calcium in these cells. These studies also suggest that spontaneous calcium events may be more prominent when neurotransmission is not reduced by the inherent disruption of the intact network in the slice preparation or by pharmacological suppression under anesthesia (Thrane et al., 2012).

A more critical challenge to the IP3 signaling dependent model of astrocytic calcium activity is the question of whether this pathway is functionally relevant to neuronal activity or synaptic transmission (Agulhon et al., 2008; Wang et al., 2009; Hamilton and Attwell, 2010). This concern stems from two recent lines of evidence. First, several studies conducted in knockouts of InsP3R2, which is the predominant InsP3R in astrocytes (Petravicz et al., 2008), did not show any differences in synaptic transmission, spontaneous firing rates or synaptic plasticity suggesting that calcium signaling through this mechanism is not necessary for these processes (Petravicz et al., 2008). The second line of evidence fuelling this controversy has come from studies using

a mouse model in which MrgA1, a non-endogenous Gq coupled receptor, was expressed within astrocytes (Agulhon et al., 2008). This system theoretically allows PLC dependent calcium within astrocytes to be triggered using a selective synthetic ligand. Although activating these receptors produced widespread calcium activity in astrocytes, it did not produce obvious changes in neuronal currents and failed to affect excitatory synaptic transmission (Fiacco et al., 2007). In a separate study from the same group, the authors also failed to observe an effect of activating MrgA1 on synaptic plasticity produced by 5 trains of theta or 100 Hz stimulation (Agulhon et al., 2010). Based on these results, the authors concluded that InsP3 dependent calcium signaling was neither necessary nor sufficient to affect synaptic transmission at least within the hippocampus.

Although these studies do raise questions regarding the regulation of astrocytic calcium dependent activity, several important caveats must be considered when interpreting these results. Subsequent studies employing the InsP3R KO mouse have demonstrated that this knockout does show deficits in cholinergic-dependent forms of plasticity (Navarrete et al., 2012) suggesting that the role of this pathway might have been missed in the relatively restricted set of electrophysiological investigation which initially characterized the model. Furthermore, expression of MrgA1 does not guarantee that this receptor effectively associates with synapses and activating this receptor may therefore produce highly non-physiological calcium signaling in astrocytes thus obfuscating any effect on local calcium activity. Indeed, the authors observed highly localized calcium waves in response to bath application of the agonist for this receptor suggesting that some form of receptor clustering occurs (Fiacco et al., 2007). Because this is a non-endogenous receptor, however, there is no reason to believe that it is

clustered adjacent to synapses. If the MrgA1 receptor is not positioned adjacent to synapses then it would be unable to induce synaptically localized calcium elevations, which several studies suggest are more directly relevant to the regulation of synaptic transmission (Di Castro et al., 2011; Shigetomi et al., 2013a). It is also important to note that, despite the observation of only subtle effects produced by activating this receptor *in situ* (Wang et al., 2013), activation of the receptor globally *in vivo* produces marked effects on depressive-like behaviors that appear to be mediated via ATP (Cao et al., 2013). Although mechanistic data are needed to corroborate these results, they do suggest that activating IP_3 dependent calcium mobilization in astrocytes can, in fact, produce an effect on CNS function.

In contrast to the negative findings from the MrgA1 and InsP3R KO models, investigations using more directly targeted, conditional approaches have provided evidence for the capacity of IP_3 dependent calcium signaling to influence synaptic transmission and plasticity. To directly assess the impact of astrocytic calcium signaling through this pathway, several studies have used uncaging of either IP_3 or calcium itself. Uncaging IP_3 *in situ* is sufficient to produce glutamate mediated SICs (Araque et al., 2000) and to modulate synaptic transmission (Perea and Araque, 2007, 2010). In addition, elevating calcium in astrocytes is sufficient to increase the frequency of miniature excitatory post synaptic currents (mEPSCs) under culture conditions. A similar increase in mEPSC frequency can be produced *in situ* by generating calcium waves in astrocytes via uncaging of IP_3 (Fiacco et al., 2007). Conversely, "clamping" calcium signaling using intracellular chelators affects synaptic plasticity, preventing LTP by reducing extracellular D-serine (Henneberger et al., 2010). In addition, a recent study

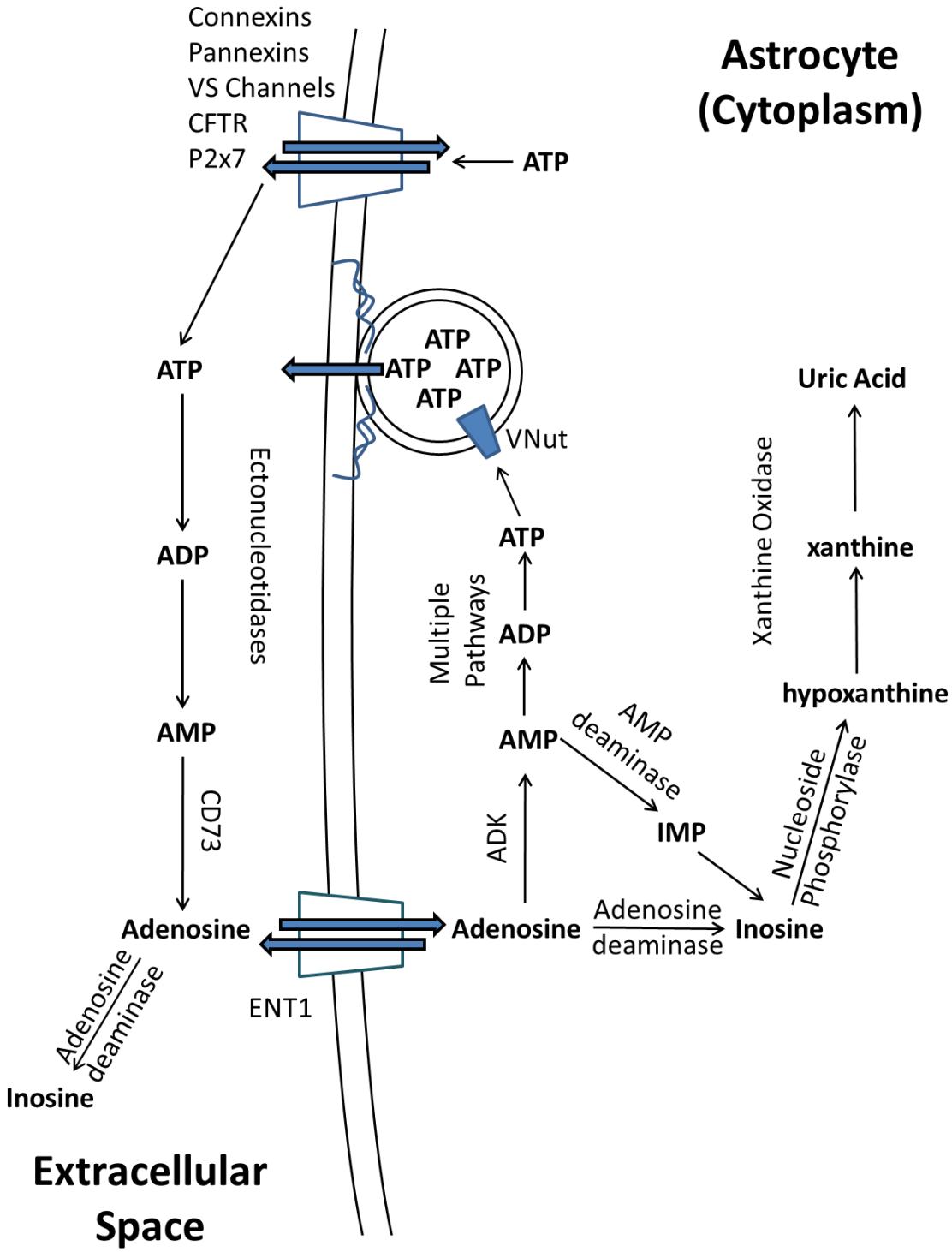
employing expression of a fragment of the InsP3 receptor to act as a high affinity IP_3 "sponge" (Tanaka et al., 2013) demonstrated that inactivating IP_3 signaling through this mechanism disrupted astrocytic morphological plasticity. The expression of this "sponge" was conditionally driven in astrocytes via a TetO transactivator system driven under the GLT-1 promoter, providing a means to conditionally disrupt IP3 in astrocytes and to avoid potential comfounds of expression during development. Although this manipulation did not produce gross structural abnormalities, electron micrographs of hippocampal synaptic structures showed that it did reduce synaptic coverage by astrocytes. In addition, mice in which IP3 signaling was disrupted by this manipulation demonstrated spatial memory deficits which the authors attributed to deficits in glutamate clearance which they observed *in situ* (Tanaka et al., 2013). Together, these results suggest that InsP3R dependent calcium signaling in astrocytes plays an important role in regulating synaptic transmission. This role may depend, in part, on controlling release of gliotransmitters from these cells. Adenosine is among the key transmitters thought to be controlled via astrocytic gliotransmission (Blutstein and Haydon, 2013). However, the mechanism by which astrocytes control extracellular levels of this molecule and its effects on synaptic transmission are incompletely understood.

Section 1.3: Multiple Cellular Sources of Extracellular Adenosine: Active Release and Concentration Dependent Control Mechanisms

Extracellular adenosine is a key neuromodulator under both physiological and pathological conditions (Ribeiro et al., 2002; Basheer et al., 2004; Fields and Burnstock, 2006; Sperlágh and Vizi, 2011; Boison, 2012). It is perhaps unsurprising, then, that

extracellular adenosine levels are controlled through multiple pathways involving release as well as clearance (Cunha, 2008; Blutstein and Haydon, 2013; Boison, 2013). In contrast to the majority of small molecule neurotransmitters, it is unclear whether vesicular release from neurons is the dominant source of extracellular adenosine (Sperlágh and Vizi, 2011). Neuronal release of this signaling molecule is known to occur under conditions of elevated activity such as those produced by application of ionotropic glutamate receptor agonist N-methyl-D-aspartate (NMDA) (Craig and White, 1993). It is unclear, however, whether this release was mediated by vesicular fusion. A more conclusive study by Klyuch et al. recently demonstrated the presence of action-potential dependent release of adenosine from parallel fibers within the cerebellum (Klyuch et al., 2011). This release was sensitive to bafilomycin, which reduces vesicular loading by inhibiting V-type H^+-ATPases (Forgac, 1999), indicating that adenosine was released through exocytosis (Klyuch et al., 2011). Despite these instances, it remains uncertain to what extent vesicular neuronal adenosine release contributes to tonic adenosine under normal physiological conditions outside of the cerebellum. In addition, further investigation of how loading of this transmitter occurs is needed, since a neuronal vesicular adenosine transporter has yet to be identified (Klyuch et al., 2012). Because the concentration of cytosolic adenosine is relatively low under physiological conditions, vesicular loading would be quite inefficient. Thus, even if vesicular release is possible it would likely lead to depletion upon repeated activation (Lovatt et al., 2012) making it difficult to maintain a high level of tonic adenosine using this pathway alone. These factors have led to a model in which adenosine levels are primarily controlled by

Figure 1.1: Adenosine Release and Clearance Pathways



astrocytes through a combination of active/passive release mechanisms of adenosine precursors and passive reuptake (Fig. 1.1)

In many cells within the CNS, the level of cytosolic adenosine is controlled by the enzyme adenosine kinase (ADK) which efficiently phosphorylates adenosine, converting it to 5'-adenosine monophosphate (AMP) (Boison, 2013). Following this initial conversion, AMP can be further phosphorylated to adenosine diphosphate (ADP) and ultimately to adenosine triphosphate (ATP)(Blutstein and Haydon, 2013). Alternatively, it can be deaminated to produce inosine monophosphate (IMP) through the calcium sensitive enzyme AMP deaminase (Mahnke and Sabina, 2005). The low level of cytosolic adenosine maintained by ADK provides the major mechanism of clearance for extracellular adenosine which tends to flow into the cell down its concentration gradient through equilibrative nucleoside transporters (ENTs). Although recent studies have demonstrated that mRNA encoding concentrative nucleoside transporters (CNTs) are present in neurons and astrocytes (Li et al., 2013) these transporters appear to contribute only slightly to the removal of extracellular adenosine. Blocking ENT-1, the primary equilibrative adenosine transporter in the adult brain, produces a large increase in adenosine mediated inhibition suggesting that the rate of clearance by CNTs alone is not sufficient to compensate for the loss of clearance by passive transport (Pascual et al., 2005; Martín et al., 2007; Etherington et al., 2009).

During development, ADK is expressed at high levels in both neurons and astrocytes. In the adult brain, however, it is primarily restricted to astrocytes, indicating that these cells provide the major "sink" for adenosine (Studer et al., 2006). Although ADK can be inhibited by substrate binding to a regulatory site outside of the enzyme's

catalytic region (Pelicano et al., 1997) it is not known to be regulated by post-translational modification and thus cannot control adenosine levels over short timescales (Fisher and Newsholme, 1984). Nonetheless, this pathway provides a potential link between adenosine signaling and cellular metabolic activity, since ATP levels depend on oxidative metabolism. In addition, recent studies from the Boison lab and others have demonstrated that expression levels of ADK are dynamically regulated under normal and pathological conditions (Boison, 2013). Following various types of brain insult, including epilepsy, traumatic brain injury and ischemia, ADK follows a biphasic pattern of expression (Boison, 2013). Under conditions of pathologically high metabolic demand, ADK is acutely downregulated and adenosine levels rise rapidly, providing a neuroprotective response to reduce excitotoxicity (Gouder et al. 2004, Pignataro et al. 2008). In the subsequent days following insult, astrogliosis occurs, leading to an overexpression of ADK and an increase in the rate of clearance of extracellular adenosine (Gouder et al. 2004, Pignataro et al. 2008). The decrease in adenosine resulting from higher reuptake is thought to contribute to the progression of brain injury and may also play a role in abnormal sleep associated with these conditions (Boison, 2013). In line with this hypothesis transgenic overexpression of the cytoplasmic isoform of ADK (ADK-tg) produces marked reduction in sleep and changes in EEG power consistent with reduced sleep pressure (Palchykova et al. 2010).

The observation that blocking passive membrane transport of adenosine increases inhibition by adorA1 receptors (Pascual et al. 2005) is consistent with a high level of ADK activity which would effectively remove adenosine from the cytosol. This strongly argues against the view that extracellular adenosine is passively released from astrocytes.

The relatively low level of ADK in neurons, however, leaves open the possibility that adenosine might be released from these cells via passive transport. A recent study employing amperometric biosensors to directly measure adenosine levels has demonstrated that neuronal release of adenosine generated by neuronal metabolic activity can indeed provide a major source of adenosine (Wall and Dale, 2013). This release occurred following stimulation of axons within the hippocampus and was mediated by transport through ENT1. Although this release was rapid and activity dependent, it did not account for a major portion of the adenosine released in response to stimulation (Wall and Dale, 2013) suggesting that multiple mechanisms control activity dependent adenosine release. This second, slower component of the adenosine response was reduced in the absence of the 5'-ectonucleotidase CD73 suggesting that conversion of metabolic precursors of adenosine might be involved in this response. In particular, the authors suggested that ATP might be converted to adenosine to produce an increase in the concentration of this molecule in response to neuronal activity. Indeed, there are several lines of evidence that indicate that extracellular hydrolysis of ATP provides a major source of extracellular adenosine (Frenguelli et al., 2007). Previous studies have suggested that this ATP is predominantly released from astrocytes, rather than neurons (Fellin et al., 2006; Halassa et al., 2009a; Blutstein and Haydon, 2013) (See Fig. 1.1).

Astrocytes are known be capable of releasing ATP through a calcium dependent mechanism both in vitro (Cornell-Bell et al., 1990; Guthrie et al., 1999) and in situ (Pascual et al., 2005). Once released into the extracellular space, ATP can signal directly through purinergic ionotropic (P_{2x}) and GPCRs (P_{2y}). This molecule is relatively unstable, however, and thus its signaling capacity is limited by the rate at which it is

hydrolyzed to downstream metabolites (Dunwiddie et al., 1997). Within the interstitial space of the CNS, ATP is primarily converted first to ADP, then to AMP and finally to adenosine. The last step in this pathway is rate-limiting and is mediated by one of a number of 5'-ectonucleotidases. In the CNS, CD73 is the primary enzyme catalyzing this reaction. This protein is ubiquitous throughout the CNS facilitating efficient production of adenosine from ATP (Dunwiddie et al., 1997). Indeed, application of exogenous ATP to hippocampal slices causes a rapid suppression of synaptic transmission which is blocked by the adorA1 receptor antagonist 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) but not by antagonists of either P_{2x} or P_{2y} receptors (Pascual et al. 2005, REF). More conclusive evidence that endogenous, tonic adenosine is derived from ATP has recently been provided by Frenguelli et al. using enzymatic biosensors to detect adenosine levels amperometrically. In this study, the authors determined that under ischemic conditions, adenosine rose rapidly. Application of the CD73 inhibitor ARL67156 eliminated a component of this release but did not completely prevent elevation of adenosine suggesting that direct adenosine release made up the majority of this response (Frenguelli et al., 2007). Importantly, however, when they applied this inhibitor under basal conditions it produced a significant reduction in the level of extracellular adenosine (110 ± 49 nmol/L) indicating that a significant fraction of tonic adenosine is derived from ATP.

Several mechanisms have been proposed to mediate release of ATP from both astrocytes and neurons (Fellin and Carmignoto, 2004; Pangrsic et al., 2007; Ben Achour and Pascual, 2012) (See Fig. 1.1). These include passive release through connexin (Cx43) and pannexin(1) channels (Arcuino et al., 2002; Heinrich et al., 2012), P_{2x7}

receptor channels (Heinrich et al., 2012), volume sensitive anion channels (Darby et al., 2003) or the cystic fibrosis transmembrane conductance regulator protein (CFTR) (Kanno and Nishizaki, 2011) as well as active release through either lysosomal or vesicular exocytosis (See Fig. 1.1). Because ATP is more highly concentrated within the cytosol relative to the extracellular space, passive release can provide a source of this molecule under basal physiological conditions. In addition, vesicular loading via the vesicular transporter Slc17A9 is relatively effective leading to high saturating concentration in vesicles (Sawada et al., 2008). In addition, since the concentration of ATP is much higher in the cell than in the extracellular space, passive diffusion of ATP out of the cell can provide a source of adenosine despite the fact that this diffusion is against the concentration gradient of adenosine itself. Although all of these mechanisms may release ATP to various degrees under different conditions, recent investigation employing molecular genetic approaches provided compelling evidence that astrocytic vesicular exocytosis is the major source of extracellular adenosine in the CNS.

Multiple studies have demonstrated that astrocytes express all of the molecular machinery necessary for SNARE dependent vesicular release and that they are capable of this form of exocytosis both in culture and *in situ* (Parpura et al., 1994a; Parpura and Zorec, 2010; Martineau et al., 2013). Several lines of evidence indicate that ATP is among the key gliotransmitters released through this mechanism and that signaling through this pathway is important for intercellular signaling to other astrocytes and neurons (Halassa et al., 2009a). Early results identified calcium dependent release of ATP as an important component of the propagation of calcium waves in astrocytic networks in vitro (Guthrie et al., 1999). Although these early studies did not determine

the precise mechanism of ATP release, subsequent investigation showed that ATP is present in vesicular granules within astrocytes and that it could be released from these cells. The authors further showed that ATP release was calcium dependent and was blocked by tetanus toxin, suggesting that the release was exocytotic through a vesicular associated membrane protein (VAMP)-dependent mechanism (Coco et al., 2003). More recently, Pangrsic et al. elegantly demonstrated vesicular fusion and quantal release of ATP by employing ATP "sniffer cells". This technology is based on HEK-293T cells expressing a mutant P_{2x3} receptor which binds with a high affinity to ATP allowing sensitive detection of this molecule in the extracellular space. By plating these "sniffer cells" adjacent to astrocytes and recording their electrical activity, the authors were able to measure periodic inward currents corresponding to release of fixed quantities of ATP by astrocytes. These events were calcium dependent and their frequency was increased by glutamate, suggesting both that this form of release occurred through vesicular fusion and that it might be activated via activation of astrocytic glutamate receptors (Pangrsic et al., 2007).

In order to determine whether astrocytic SNARE dependent vesicular release of ATP provides the major source of adenosine *in situ*, Pascual et al. created a transgenic mouse model, the "dnSNARE" mouse, in which SNARE-mediated fusion is disrupted in astrocytes (Pascual et al., 2005). To accomplish this, the authors developed a novel system allowing conditional expression of a cytosolic fragment of the protein VAMP-2 (previously called synaptobrevin-2) which acts in a dominant negative manner to disrupt SNARE complex formation (dnSNARE). The authors then used the "tet-Off" tetracycline transactivator under control of the astrocyte specific glial fibrillary acidic

protein (GFAP) promoter to drive expression of the dnSNARE transgene along with the fluorescent reporter enhanced green fluorescent protein (eGFP). This system allows astrocyte specific expression of dnSNARE to be inhibited by doxycycline during development, thus preventing any potentially confounding developmental effects. In hippocampal slices taken from these mice application of the adorA1 antagonist produces a relatively minor enhancement of synaptic transmission, demonstrating that this transgene significantly reduces the level of presynaptic inhibition by adorA1 receptors. Because no corresponding change in sensitivity to adorA1 receptor agonists was observed, this indicates that disrupting astrocytic exocytosis leads to a reduction in extracellular adenosine. The authors also observed that inhibiting ectonucleotidases, which typically increases inhibition via P_{2y} receptors by blocking the conversion of ATP to adenosine, produced no effect in dnSNARE mice. This finding shows that the lower level of tonic adenosine corresponds to reduced release of ATP suggesting that vesicular release of ATP might be the source of adenosine which is disrupted by dnSNARE expression. The authors provided further support for this idea using luciferase based assays which directly measured levels of ATP. These experiments revealed a reduction in the extracellular concentration of ATP within hippocampus of dnSNARE mice relative to controls suggesting that tonic release of this molecule was reduced.

Recent results have further demonstrated that the involvement of SNARE mediated exocytosis is not only a source of tonic adenosine but also plays a role in activity-dependent elevation of adenosine. In this study, the authors employed direct measurement of extracellular adenosine along with the dnSNARE transgenic to show that approximately 40% of the adenosine released by stimulating the Schaeffer collateral

pathway was due to SNARE dependent release from astrocytes (Wall and Dale, 2013). Using a CD73 KO, the authors further demonstrated that this pool of activity-dependent adenosine arose from conversion of AMP to adenosine (Wall and Dale, 2013). Taken together, these results indicate that astrocytes provide a major source of tonic and activity dependent inhibition through exocytosis of ATP which is subsequently metabolized to adenosine. *Despite this evidence, however, physiological stimuli capable of producing changes in astrocyte-derived adenosine have not been identified.* ***In chapter 5, I demonstrate that astrocytes can increase the concentration of extracellular adenosine through a calcium dependent mechanism and that this elevation depends on vesicular release, either of adenosine or of one of its metabolic precursors.***

Section 1.4: The Role of Astrocyte-Derived Adenosine in Synaptic Plasticity

Several functions have been ascribed to astrocyte-derived adenosine in the regulation of hippocampal synaptic transmission. One of these effects is a form of short term plasticity mediated by astrocytes known as heterosynaptic depression. This phenomenon is thought to be based on the unique capacity of astrocytes to regulate multiple adjacent synapses within a single astrocytic territory (Zhang et al., 2003; Halassa et al., 2007b; Cunha, 2008). Heterosynaptic depression was initially observed in stratum radiatum of hippocampal area cornu-amonis area 1 (CA1). It occurs in response to tetanic stimulation of axons in this pathway leading to presynaptic inhibition of adjacent synapses to which these axons did not project (Manzoni et al., 1994). This effect is blocked by adorA1 receptor antagonists and a similar inhibition can be induced by exogenous application of adenosine, suggesting that adenosine signaling is involved in

this phenomenon (Zhang et al., 2003). In addition, this effect is blocked by disruption of gliotransmission in the dnSNARE mouse model, suggesting that astrocytic release of ATP provides the source of the inhibitory adenosine (Pascual et al., 2005).

The involvement of astrocytic exocytosis in heterosynaptic depression has been challenged by a recent study which argued that direct neuronal release of adenosine was responsible for heterosynaptic inhibition (Lovatt et al., 2012). The contention of this study is based on the observation that double knockouts of CD73 which, as previously stated, is the major enzyme responsible for the conversion of AMP, still show heterosynaptic depression under appropriate stimulation conditions. This suggests that conversion of AMP to adenosine is not necessary for this form of plasticity and thus that ATP release need not be responsible for the increase in adorA1 receptor mediated inhibition by this stimulation protocol. In a recent study by Rittiner and colleagues, however, the authors clearly establish that AMP is capable of directly activating adorA1 receptors suggesting that metabolic intermediates can fulfill the role of adenosine even in the absence of complete ATP hydrolysis (Rittiner et al., 2012). This leaves open the possibility that astrocyte-derived AMP might play the role normally occupied by adenosine in the CD73 double knockout and argues against the assertion that astrocytic release does not play a role in heterosynaptic depression even in the absence of this enzyme (Blutstein and Haydon, 2013).

In addition to identifying its role in heterosynaptic depression, Pascual and colleagues also demonstrated that astrocytic gliotransmission is capable of regulating long term potentiation (LTP) induced by theta burst stimulation (Pascual et al., 2005). This form of stimulation mimics activity observed during learning behavior

(Vinogradova, 1995; Kahana et al., 1999) and is considered an effective means to investigate modulation of synaptic potentiation important for hippocampal memory (Nguyen and Kandel, 1997). In dnSNARE mice, LTP is reduced relative to control under baseline conditions. This effect was mimicked by application of the adorA1 receptor antagonist DPCPX suggesting that it was mediated by astrocyte-derived adenosine (Pascual et al., 2005). Although the authors did not determine the origin of this effect, it probably involves the reduced NMDA receptor surface expression observed in dnSNARE mice (Deng et al., 2011) since these receptors are critically involved in theta burst induced LTP within the area they were recording, area CA1 of the hippocampus (Abel et al., 1997; Nguyen and Kandel, 1997). Alternatively, the effect may occur because of changes in the vesicular release of D-serine. This gliotransmitter, whose release is also reduced by dnSNARE expression (Pascual et al., 2005; Panatier et al., 2006; Martineau et al., 2013), has been shown to act as an essential co-agonist at synaptic NMDA receptors so lower levels in the extracellular space would produce a deficit in synaptic plasticity (Papouin et al., 2012).

Given the observed reduction in LTP, one might expect that dnSNARE mice would show a deficit in hippocampal dependent memory relative to WT controls. Under undisturbed conditions, however, these mice show no difference in learning or memory consolidation in the novel object (Halassa et al., 2009b) or spatial novel object (Florian et al., 2011) tasks, both of which are thought to depend to some extent on plasticity within the hippocampus (Squire and Zola-morgan, 1978). The effect of inhibiting gliotransmission on cortical activity and sleep need is only apparent following sleep deprivation. This implies that astrocytes may provide broadly homeostatic regulation of

synaptic transmission which may not be apparent under baseline conditions but may instead require some form of perturbation such as sleep loss. Indeed, given the relationship between astrocytes and the regulation of sleep coupled with the importance of sleep for cognitive function, it seems quite possible that gliotransmission might specifically affect sleep related changes in CNS function. Nonetheless, the role of astrocytes in this process remains incompletely understood. In addition, *the possibility that astrocyte derived adenosine can modify synaptic plasticity or inhibit synaptic transmission in response to activation of calcium dependent release has not been previously tested.* ***In the fifth chapter of this dissertation I show that triggering astrocyte-dependent elevation of adenosine leads to increases in NMDA receptor mediated synaptic transmission as well as presynaptic inhibition at higher levels of activation. I also demonstrate that activation of adorA1 receptors leads to enhanced synaptic plasticity for low threshold induction of long term potentiation.***

Section 1.5: Wakefulness-Dependent Changes in Adenosine Regulate Sleep

Extracellular adenosine is a major modulator of neurotransmission in the central nervous system (CNS) and is also involved in multiple regulatory pathways in the periphery which will not be considered here (for review see Ralevic & Burnstock, 1998). In the brain it can act through several classes of GPCRs including Gi coupled adorA1 receptors and Gs coupled adorA2 receptors. These two receptor types are thought to be the primary means by which extracellular adenosine acts within the CNS. The adorA2 receptor is further subdivided into two classes, the adorA2a and adorA2b receptors which have distinct functions and different patterns of expression (Dunwiddie and Masino,

2001). Among these receptor subtypes, the relative affinity for adenosine is highest for the adorA1 and adorA2a receptor subtypes and of these only the adorA1 receptor is broadly expressed at high levels in multiple brain regions (Fastbom et al., 1987; Elmenhorst et al., 2007). In contrast, expression of the adorA2a receptor within the brain is primarily restricted to the striatum and nucleus accumbens. Classically, activation of the adorA1 is thought to primarily reduce neuronal excitability by hyperpolarizing neurons and also by reducing vesicular release (Dunwiddie and Fredholm, 1989). This pathway plays a key role in neuroprotection under pathological conditions such as seizure and stroke during which the increase in intracellular adenosine causes large scale release through the passive equilibrative nucleoside transporters (ENTs) leading to widespread inhibition (Latini and Pedata, 2001). Under physiological conditions, however, adenosine is taken up by ENT1 and blocking these transporters increases activation of adenosine receptors (Pascual et al., 2005; Martín et al., 2007) as well as levels of extracellular adenosine (Frenguelli et al., 2007). This suggests that the level of adenosine is higher in the extracellular space than in the cytosol indicating that other release mechanisms with potentially distinct physiological functions are at play during normal brain function.

In addition to its involvement in neuroprotection, several lines of evidence have implicated adenosine as the major mediator of the homeostatic sleep drive in mammals (Porkka-Heiskanen, 1997; Díaz-Muñoz and Salín-Pascual, 2010; Blutstein and Haydon, 2013) and more broadly as a key player in synchronizing neuronal network activity. Indeed caffeine, the most commonly used psychoactive substance, is thought to produce its wakefulness-promoting and stimulant effects primarily by blocking adorA1 and

adorA2a receptors (Ribeiro et al., 2002). Conversely, adenosine receptor agonists promote sleep when provided centrally. This effect is thought to be at least partially mediated by adorA1 receptors on acetylcholine neurons projecting to the cortex from within the basal forebrain (BF, Brown et al., 2012) and from other cholinergic neurons located in the laterodorsal tegmental nucleus (LDT). Cholinergic projections from these nuclei are a key part of the ascending arousal system responsible for maintaining the desynchronized patterns of brain activity associated with wakefulness and rapid eye movement (REM) sleep. Consistent with the proposed role of adenosine in regulating these nuclei, microdialysis based measurement in the BF and cortical regions to which it projects show that levels of adenosine follow a diurnal rhythm (Murillo-Rodriguez et al., 2004) and increase during sleep deprivation (Kalinchuk et al., 2011). Although the mechanism of wakefulness dependent changes in adenosine levels in these brain nuclei are incompletely understood, recent *in situ* (Sims et al., 2013) and *in vivo* (Kalinchuk et al., 2010) studies have shown that changes in adenosine within these regions involve nitric oxide production by inducible nitric oxide synthase.

Adenosine is thought to produce its inhibitory effects on multiple classes of neurons, including cholinergic, wake-promoting neurons of the BF, by activating adorA1 receptors leading to reduced firing rates and inhibition of synaptic release. Within the BF, application of the adorA1 receptor agonist N^6-cyclohexyladenosine (CHA) produces a reduction in single unit activity of wake active neurons suggesting that activation of this receptor is sufficient to directly suppress wake-promoting patterns of activity in this nucleus (Alam et al., 1999). Conversely, application of the selective adorA1 antagonist cyclopentyl-1,3-dimethylxanthine (CPT) or knockdown of A1 receptors using antisense

oligonucleotides in this region promotes wakefulness and increases firing rates of wake-active neurons (Strecker et al., 2000; Thakkar et al., 2003). Cortical delta activity is a well-established marker of sleep pressure which increases with progressive wakefulness and is associated with lower sleep latency following sleep deprivation. Knocking down adorA1 receptors in the BF also prevents sleep deprivation associated increases in delta activity (Thakkar et al., 2003). In addition, conditional knockout of adorA1 receptors in Calcium Kinase II expressing neurons also led to significant deficits in sleep homeostasis (Bjorness et al., 2009), providing additional evidence for the importance of the receptor in this system.

The effect of adenosine on firing rates of wake active neurons in the BF is thought to be primarily mediated by direct modulation of neuronal excitability through somatic adorA1 receptors. Studies from Arrigoni and others have demonstrated that this effect is due to hyperpolarization (Strecker et al., 2000) and increased shunting inhibition produced by activating G-protein coupled inward rectifying potassium channels (GIRKs, Trussell and Jackson, 1985). The magnocellular preoptic nucleus and substantia inominata project broadly to the cortical mantle and hippocampus and are considered the main wake active nuclei of the BF. Application of adenosine and adorA1 agonists to these areas has been shown to activate an inwardly rectifying potassium current with a reversal potential of -82 mV (Arrigoni et al., 2001). This current is present in cholinergic neurons but absent in putative GABAergic interneurons which instead display reduced firing rate due to inhibition of the hyperpolarization activated cation-current normally present in this cell type (Arrigoni et al., 2006). Activation of the inward rectifying potassium current by adorA1 receptors is common to many groups of pyramidal cells

throughout the cortex and varies systematically with passive membrane properties (van Aerde et al., 2013), suggesting that it plays a role in inhibiting excitatory neurotransmission within a variety of circuits (Porkka-Heiskanen and Kalinchuk, 2011). Interestingly, however, the effect observed in some non-cholinergic neurons appears to be absent even in other, classes of similar neurons. This is even true of some non-cholinergic neurons within the BF (Arrigoni et al., 2006). In addition, the hyperpolarizing effect is not observed in many types of cortical neurons, suggesting that modulation by adenosine may play a variety of distinct roles beyond its canonical somatic inhibitory effect.

Adenosine can inhibit wakefulness promoting nuclei in the LDT and BF through two pathways. First, as described above, it can reduce firing rate by acting on somatic adorA1 receptors within the nuclei leading to hyperpolarization. Secondly, adenosine can inhibit acetylcholine release from BF and LDT projections onto cortical and thalamic neurons, respectively (Sperlágh and Vizi, 2011; Brown et al., 2012). Adenosine can slowly rise in the cortex following extended sleep deprivation, suggesting that presynaptic inhibition in the cortex may also be involved in promoting sleep. This idea is supported by a recent study which demonstrated that local application of adorA1 receptor agonists in the prefrontal cortex, an area that is not essential for sleep cycle generation, is sufficient to decrease arousal and increase slow wave activity (Van Dort et al., 2009). The effect of this agonist was also associated with a reduction in acetylcholine suggesting that it was still due to inhibition of BF projecting neurons, in this case through a reduction of transmitter release. AdorA1 receptors produce this effect by decreasing cyclicAMP production resulting in reduced sensitivity of voltage gated calcium channels

to depolarization. Further evidence for the role of this type of presynaptic inhibition in the sleep inducing effects of adenosine comes from studies of mice expressing a mutant form of presynaptic, voltage gated calcium channel ($Ca_v2.1$, Deboer, van Diepen, Ferrari, Van den Maagdenberg, & Meijer, 2013). This mutant, known as Cacna1a R192Q, expresses a version of $Ca_v2.1$ that is less effectively modulated by changes in cyclicAMP. These mice are less sensitive to the sleep promoting effects of adorA1 agonist and show reduced sleep pressure in response to sleep deprivation (Deboer et al., 2013).

In the human brain, positron emission tomography (PET) has been used to indirectly examine the level of adenosine receptors based on the relative binding of radioligands that have high affinity for the adorA1 receptor, [^{18}F]8-cyclopentyl-3-(3-fluoropropyl)-1-popylxanthine ([^{18}F]CPFPX). Studies using this technique observe a net increase in binding by this radioligand in response to sleep deprivation, suggesting an up-regulation of adenosine receptor surface expression (Elmenhorst et al., 2007). This increase is likely due to higher transcription of the adorA1 receptor, since sleep deprivation increases the level of messenger RNA (mRNA) encoding this receptor type in neurons and glia in rodent studies (Basheer et al., 2001). The elevation in receptor levels may compensate for receptor internalization occurring after prolonged adenosinergic activation (Klaasse et al., 2008). However, the precise function of this positive feedback mechanism remains incompletely understood. In addition, although this change is detectable in multiple parts of the cortex as well as the thalamus, striatum and cerebellum, it varies as a function of brain region providing further evidence that the effect of adenosine signaling on transcription is variable based on which circuits are involved (Cunha, 2008).

Together, these findings show that wakefulness-dependent changes in adenosine signaling occur broadly throughout the CNS to promote sleep. They also suggest, however, that the effects produced by this transmitter can vary depending on the brain region, cell type and the subcellular localization of the receptor. The variation in the effects of activating adorA1 receptor is based on the fact that it can couple to multiple downstream signaling pathways leading to distinct responses (Cordeaux et al., 2004). This variety is matched by a similar diversity in the active and passive sources. *While wakefulness-dependent regulation of adenosine in the extracellular space can control multiple sleep related behaviors, the source of the increased adenosine associated with sleep-loss remains unknown.* ***In chapter 3, I show that prior wakefulness induces an increase in extracellular adenosine and that this increase requires SNARE dependent vesicular release from astrocytes.***

Section 1.6: Involvement of Adenosine Signaling in the Effect of Long Term Sleep Disruption on Sleep Homeostasis and Alcohol Sensitivity

Sleep disorders are prevalent and account for U.S. $15.9 billion annually in lost productivity and higher health care costs (National Center for Sleep Disorders). Treatments for sleep disorders have variable efficacies, most likely resulting from the multitude of environmental and genetic variables that are known to influence the development of chronic sleep impairments. For the same reason, it has been difficult to identify the cellular mechanisms that contribute to sleep impairments. Sleep is regulated by two fundamental processes—a circadian process that is entrained by environmental factors such as light or food, and a homeostatic process, which provides the drive for

sleep as a function of prior wakefulness (sleep pressure; Borbély & Achermann, 1999). Although these two components of the sleep regulatory machinery depend on completely separate mechanisms they interact in complex ways and these interactions are critically responsible for the control of sleep drive.

A complete discussion of the circadian regulation of sleep is beyond the scope of this dissertation. Because it provides an important component of the sleep regulatory system, however, I will briefly summarize key findings in this field. First, it is important to note that circadian rhythm is governed at the cellular level by the oscillation of intrinsically cycling gene products which do not require synaptic input to maintain their pattern of expression. This molecular circadian oscillator consists of a feedback loop made up of a set of core genes that indirectly regulate their own expression. The first part of this feedback loop consists of the positive transcriptional regulators brain and muscle ARNT-like1 (BMAL1) and a mammalian protein known as CLOCK (CLK). The proteins encoded by these genes are chromatin remodeling DNA binding proteins which drive expression of negative regulatory proteins cryptochrome (CRY) and period (PER)(Pace-Schott and Hobson, 2002; Franken and Dijk, 2009). These negative regulatory proteins then bind to the BMAL1/CLOCK complex to inhibit their own transcription. When peak expression of the negative regulatory component of the oscillator prevents further expression of CRY and PER, the level of these proteins begins to fall due to degradation through post-translational modification and proteosomal degradation. This allows BMAL1/CLOCK to drive transcription, beginning the cycle anew. The output of this core molecular oscillator is provided by the superchiasmatic nucleus which acts as a central pacemaker to govern behavioral rhythms via hormonal

release and direct projects to wake/sleep active nuclei (Saper et al., 2010). Changes in light onset and the timing of food availability reset this rhythm, allowing it to be entrained, to local conditions. This entrainment, occurring in response to long distance flights (jetlag), for example, is often acute and adaptive (Saper et al., 2010) providing a means to adapt to changes in the environment. Interestingly, some recent studies have suggested that protein levels of many of the enzymes which regulate extracellular adenosine vary diurnally (Mackiewicz et al., 2003). Although this study did not conclusively determine whether the variation was controlled by circadian transcription factors, they did observe that expression of these enzymes was not driven by wakefulness, suggesting that the variation might be controlled by the biological clock.

Despite the tight regulation of sleep by circadian and homeostatic processes, chronic sleep disruption is a frequent occurrence in modern life, often occurring in association with work-related demands such as completing major research documents. This protracted restriction of sleep time can lead to long-lasting impairments in cognitive function (Banks & Dinges, 2007; for review see McCoy & Strecker, 2011). For example, chronic sleep loss has been shown to induce sustained cognitive impairments, despite subjective reporting of sleepiness dissipating over time (Van Dongen et al., 2003). In addition, some studies have reported that an allostatic reduction of sleep pressure occurs following prolonged periodic sleep loss (Kim et al., 2013). These results have not been observed in all cases. One study, which measured slow wave power in separate brain regions, failed to observe a similar effect (Leemburg et al., 2010). This inconsistency may be due to differences in methodology in the recording system or to differing methods of analysis since, in the latter study, region specific activity for each quadrant of the brain

was isolated using multiple electrode signals. Because slow wave activity can be generated locally as well as propagating between cortical areas (Vyazovskiy et al., 2011), one possible explanation is that local slow wave activity is regulated normally whereas propagation across cortical areas is modified. In any case, if they prove correct, these results suggest that the sleep homeostat is differentially affected by longer versus shorter periods of deprivation. To date, however, the mechanism underlying this consequence of chronic sleep restriction remains unknown.

In addition to their direct effects on cognition, impairments in sleep homeostasis are frequently associated with several psychiatric conditions including substance abuse and dependence (Brower, 2003; Armitage, 2007; Wong et al., 2009; Wulff et al., 2010). Alcoholism, in particular, is strongly associated with sleep disruptions. Childhood sleep disturbances strongly predict an increased propensity to abuse alcohol later in life (Wong et al., 2010). In addition, alcohol is often used as self-medication among insomniacs (Brower, 2003). Sleep disturbances are especially severe in recovering alcoholics and are a primary contributor to relapse (Brower et al., 2011). In part because of the complex pharmacology of alcohol, however, the influence of sleep disruptions on the behavioral effect of alcohol, though prominent, remain poorly understood (Ruby et al., 2010). Alcohol can affect the clearance of adenosine, suggesting the possibility that this signaling molecule provides the nexus point for the interaction between alcohol addiction and sleep regulation. This hypothesis further implies that astrocytes may be involved in regulating the effects of alcohol on sleep function, potentially through changes in release of adenosine in response. *It remains unknown whether astrocytic gliotransmission is involved in alcohol dependence or in any other sleep related neurological disorders.* ***In***

Chapter 4 of this dissertation, I demonstrate that long term sleep restriction leads to a disruption of the wakefulness dependent increase in adenosine and that this reduction leads to a disruption in both sleep deprivation induced sleep pressure and to the behavioral sensitivity to alcohol.

Section 1.7: The Role of Astrocyte-Derived Adenosine in Sleep Associated Cortical Oscillations

The association between increases in extracellular adenosine and higher slow wave activity characteristic of higher sleep pressure suggests that wakefulness-dependent changes in this neuromodulator might play a role in coordinating neuronal networks. Specifically, it would imply that elevation of adenosine for extended period would lead to increased synchronization during subsequent NREM sleep. Since the activity underlying these rhythms is considered important for memory consolidation (Diekelmann and Born, 2010; Inostroza and Born, 2013; Nere et al., 2013) and memory triage (Stickgold and Walker, 2013) this implies that adenosine, and by extension the astrocytes which supply it, might be involved in regulating sleep-dependent plasticity. This idea is supported by the observation that astrocyte-derived adenosine can impact both basal synaptic transmission as well as certain forms of synaptic plasticity in situ (Pascual et al., 2005). Because of the diversity of signaling pathways through which adenosine can modulate neuronal firing rates and synaptic transmission, however, the effects of astrocytic release of this molecule on brain function have only recently begun to be understood (Halassa et al., 2007a; Halassa and Haydon, 2010).

Synchronized cortical oscillations are complex phenomena and the extent to which this form of network activity is controlled by local versus global mechanisms remains controversial. However increasing evidence indicates that astrocytes are involved in patterning this type of cortical activity (Halassa, 2011). As discussed earlier, activation of adorA1 receptors in either sleep centers, such as the BF, or directly in the prefrontal cortex is sufficient to potentiate slow wave activity and promote sleep. Because astrocytic gliotransmission provides a major source of adenosine, this suggests that exocytotic release from this cell type may be involved in coordinating network activity to promote NREM sleep as part of the homeostatic sleep response (Pascual et al., 2005). To test this hypothesis, Halassa et al. performed electroencephalographic measurements in the dnSNARE mouse under both baseline conditions and after sleep depriving the mice to increase sleep pressure. Attenuating gliotransmission with this dominant negative transgene did not affect baseline sleep properties. Interestingly, however, closer examination of the spectral power during NREM sleep revealed a reduction in the power of low frequency slow wave activity (SWA). The SWA frequency range corresponds to synchronized firing in the 0.5-1.5 Hz corresponding to up-down state firing and is a well-established indicator of sleep depth (Destexhe et al., 2007; Fellin et al., 2009; Halassa et al., 2009b). This suggests that gliotransmission does modulate neuronal activity to promote forms of network synchronization associated with sleep. Because sleep is regulated by multiple mechanisms, including circadian rhythms coordinated by the superchiasmatic nucleus (Wulff et al., 2010), under normal conditions astrocytic modulation alone is not sufficient to promote sleep.

Because the homeostatic sleep response is characterized by both an increase in recovery sleep and higher slow wave power associated with an increase in extracellular adenosine (Basheer et al., 2004), Halassa et al. next tested the effect of disrupting gliotransmission on the recovery from acute sleep deprivation. Under these conditions, the reduction in slow wave power was even more pronounced and the increase in sleep time following sleep deprivation was significantly mitigated, indicating that astrocytes play a key role in sleep homeostasis (Halassa et al., 2009b). In addition, chronic application of adenosine receptor antagonists phenocopied the effect of dnSNARE expression. Together, these results indicate that adenosine is the major gliotransmitter through which astrocytes regulate sleep homeostasis (Halassa et al., 2009a). This effect could be due to activation of adorA2a receptors since antagonists of this receptor also increase waking (Díaz-Muñoz and Salín-Pascual, 2010). Surprisingly, however, while dnSNARE occludes the wakefulness-promoting effect of adorA1R antagonists, the effects of adorA2a receptor antagonists were still observed suggesting that they were activated by adenosine from a different source. The lack of effect is particularly surprising considering the relative affinity of adorA2a receptors compared to adorA1 receptors. Because adorA2a receptors are considerably less sensitive to adenosine one might expect that the sparse expression of dnSNARE would be more likely to disrupt adorA2a activation but this is not the case. This effect may instead occur because of the difference in the distribution of the two receptor subtypes, since adorA1 receptors are widely expressed throughout the brain while adorA2a receptors are mainly expressed in the striatum (Ralevic and Burnstock, 1998). However this also seems unlikely, because dnSNARE is also expressed within the striatum. Because astrocytic dnSNARE

expression does not completely eliminate release from astrocytes, it may be that these receptors are activated by adenosine produced from the remaining pool of ATP. Although this explanation may initially seem unlikely given the relatively lower affinity of adorA2a receptors, these receptors are known to form a complex with the ectonucleotidase CD73 in the extracellular space. This connection potentially provides adorA2a receptors with a privledged source of adenosine which allows them to be less dramatically affected by the reduction in ATP release (Augusto et al., 2013).

Further investigation using direct measurement of local field potential under urethane anesthesia determined that reducing gliotransmission inhibited low frequency cortical oscillations and decreased the frequency of UP-DOWN state transitions in individual neurons (Fellin et al., 2009). This reduction was not directly due to reduced tonic adenosine because transient application of the adorA1 receptor antagonist CPT increases slow wave oscillation under these conditions rather than reducing them. Instead, the authors showed that the effect was due to reduced activation of the NMDA receptor since blocking these receptors with D-AP5 reduced slow oscillations to a greater degree in wild type (WT) mice compared with dnSNAREs. Application of exogenous D-serine, which is also known to be released from astrocytes (Panatier et al., 2006; Henneberger et al., 2010; Martineau et al., 2013), partially rescues this effect suggesting that this gliotransmitter may be involved in the reduction in slow wave oscillations. Reduced D-serine may not completely account for the observed change in LTP, however, because the surface expression of NMDAR NR2A and NR2B subunits is also reduced in dnSNARE mice (Fellin et al., 2009).

Despite the clear role of adenosine in inducing NREM sleep and promoting slow wave activity, adenosine does not directly induce slow wave oscillations in anesthetized mice. Interestingly, however, the acute effect of CPT on cortical activity was greater in control mice compared with dnSNAREs indicating that the level of tonic adenosine was still reduced in the absence of gliotransmission. The possibility remains, then, that changes in the level of adenosine can play a role in potentiating slow wave oscillations through an indirect pathway. A recent study by Deng et al. lent support to this idea by identifying a pathway through which activation of adorA1 receptors led to changes in postsynaptic receptor profile (Deng et al., 2011). Although this type of adenosine receptor is classically associated with presynaptic and somatic effects, they are highly enriched in postsynaptic fractions (Rebola et al., 2003) so they have the capacity to regulate this subcellular compartment as well. Indeed, Deng and colleagues demonstrated that the lower surface expression of NMDAR receptors observed in dnSNARE mice was rescued by incubation with the adorA1 receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA). Conversely, incubating slices in 200 nM CPT phenocopied dnSNARE by reducing NR2A and NR2B surface expression. These effects were functionally important since they led to a higher AMPA/NMDA receptor ratio as well as a decrease in the NMDA receptor components of miniature EPSCs (Deng et al., 2011). Further investigations showed that the effect of adorA1 activation was mediated through src/fyn dependent phosphorylation of NMDA receptor subunits which caused a reduction in the rate of internalization leading to greater surface stability of these receptors.

Given the importance of NMDA receptors in plasticity along with the association between slow wave activity and memory consolidation, these findings suggests that astrocyte-derived adenosine could play a role in declarative memory functions related to sleep. Although the relationship remains incompletely understood, several studies have begun to illuminate the role of this form of gliotransmission in memory consolidation. Indeed, it is increasingly clear that astrocytes play a key role in regulating synaptic transmission within the hippocampus, the major brain structure supporting spatial declarative memory (Squire and Zola-morgan, 1978). Astrocyte derived adenosine also affects cortical function leading to changes in global network activity. *However, although adenosine derived from this cell type is clearly involved in sleep homeostasis, it is unclear whether wakefulness can affect cortical network activity through this pathway.* ***In the third chapter of this dissertation, I show that the level of adenosine mediated modulation of cortical activity is affected by wakefulness history and that the mechanism underlying this change requires astrocytic SNARE mediated release.***

Section 1.8: The role of Astrocytes in Sleep-Dependent Memory Consolidation

The biological effects of sleep are multifaceted. It is clear that sleep is critically involved in brain function and that among its most essential functions is the evolution of memory during NREM and REM sleep. Insufficient sleep has been shown to disrupt hippocampal dependent memory performance in both rodents and humans (Graves et al., 2003; Yoo et al., 2007). Conversely, during sleep declarative memory is stabilized and enhanced through a process known as consolidation (Stickgold and Walker, 2007). Interestingly this stabilization is not uniform since memories that have increased

emotional valence (Hu et al., 2006) or greater behavioral relevance for future reward (Fischer and Born, 2009) show a greater degree of retention compared with less "important" memories. This has led to the concept of memory "triage", in which some hippocampal and cortical circuits that undergo plasticity during wakefulness are selectively enhanced relative to other inactive or less essential representations (Stickgold and Walker, 2013).

Several mechanisms have been proposed to explain how changes to memory during sleep occur at the cellular and synaptic level and how memory function is disrupted by sleep loss (Diekelmann et al., 2009; Diekelmann and Born, 2010). In addition to the well defined behavioral and electroencephalographic markers associated with wakefulness and sleep, there are a variety of changes in gene expression (Tononi and Cirelli, 2001; Romcy-Pereira et al., 2009; Wang and Liu, 2010), protein levels (Vyazovskiy et al., 2008; Gilestro et al., 2009), receptor surface expression (Longordo et al., 2009), synaptic structure (Bushey et al., 2011) and metabolic activity (Porkka-Heiskanen and Kalinchuk, 2011; Xie et al., 2013). In addition, sleep deprivation produces a distinct set of changes that may contribute to the deficits associated with sleep loss (Kopp et al., 2006; Wang and Liu, 2010; Havekes et al., 2012). Overall protein synthesis is promoted during sleep (Seibt et al., 2012), an effect which many authors have interpreted as a component of the restorative function of sleep and of NREM sleep in particular (Naidoo, 2009). Sleep loss has also been shown to reduce brain derived neurotrophic factor (BDNF) specifically within the hippocampus, providing further evidence that this structure is particularly sensitive to sleep disruption (Guzman-Marin et al., 2006). Although many of the changes in cellular physiology that occur during

wakefulness or sleep deprivation may not be directly related to the maintenance of hippocampal function, it is likely that some of them play a role in regulating memory.

Among the core biological effect of sleep on neurological activity is the reduction in net potentiation across synapses relative to wakefulness. Changes in the relative level of potentiation is observed after even relatively short periods of sleep (Vyazovskiy et al., 2008; Nere et al., 2013). This "synaptic down-scaling" counters the increase in ionotropic, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors in synaptic terminals during the subjective day. Because sleep deprivation further increases levels of this protein in synaptoneuronsomes and also leads to increased NMDA receptor levels at synapses, this increased net potentiation appears to be wakefulness-dependent rather than a circadian variation in surface expression (Kopp et al., 2006). Up-regulation of NMDA receptor surface expression, and in particular of NR2A containing NMDA receptors, may be at least partially responsible for deficits associated with acute sleep deprivation since NR2A knockout mice showed a reduced deficit in hippocampal plasticity following sleep loss (Longordo et al., 2009). Given the capacity of astrocyte-derived adenosine to increase the stability of NMDA receptors, this effect may be due to increased adenosine within the hippocampus although this remains to be investigated.

In addition to increasing net potentiation, extended wakefulness is known to have a variety of effects on synaptic plasticity. The molecular signaling mechanisms through which sleep deprivation controls plasticity have been intensely investigated but they remain incompletely understood. In a recent study, Vecsey and colleagues demonstrated that protein kinase A (PKA)-dependent/5'-cyclic AMP (cAMP)-dependent synaptic

plasticity are negatively affected by sleep deprivation (Vecsey et al., 2009). The authors observed that a 5-hour period of sleep loss produced deficits in multiple forms of LTP, including the form produced by theta burst stimulation, which are known to require PKA and cAMP mediated transcriptional regulation. The authors further showed that enhancing signaling through this pathway by inhibiting phosphodiesterase 4, an enzyme that degrades cAMP, rescued some of the deficits in LTP, and also improved performance in a hippocampal dependent fear conditioning task (Vecsey et al., 2009). Coupled with a meta-analysis of microarray data from multiple rodent studies taken following sleep deprivation that implicated gene network associated with cAMP mediated transcription as a key pathway specifically modified by sleep loss (Wang and Liu, 2010), these results strongly suggest that abnormal activity of this pathway is involved in the detrimental impact of enforced wakefulness.

As discussed earlier, changes in the concentration of astrocyte-derived adenosine mediate the homeostatic sleep response. The correlation between higher adenosine and the negative effects of sleep loss suggests the possibility that these deficits may involve the accumulated effect of astrocyte derived adenosine. This idea is supported by the observation that the disruption of astrocytic exocytosis in the dnSNARE mouse prevents the deficits in memory normally produced by sleep deprivation (Halassa et al., 2009b). A similar protective effect can be obtained with chronic application of CPT to the CNS suggesting that the deficits in memory consolidation associated with sleep loss require activation of adenosine receptors (Halassa et al., 2009b). Because adorA1 receptors are frequently Gi coupled this effect may be due to reduction of cAMP in the postsynaptic terminal. Consistent with this hypothesis, Florian et al. showed that the deficits in

cAMP-dependent LTP resulting from sleep deprivation were absent in dnSNARE mice and were also blocked by CPT (Florian et al., 2011). These results strongly suggest that astrocytes regulate both synaptic transmission and plasticity in the hippocampus through activation of adorA1 receptors coupled to multiple downstream signaling pathways including Gi coupled cAMP regulation. *Despite this insight, however, the precise means by which astrocytes respond to wakefulness to control synaptic plasticity is poorly understood and therefore remains a key area of investigation in which further research is required.*

In the following chapters, we investigate the impact of wakefulness-dependent signaling by astrocyte-derived adenosine on synaptic transmission and plasticity in the hippocampus. We show that astrocytes provide a major source of this transmitter and that they are capable of controlling its extracellular concentration in response to wakefulness. We further show that astrocytes allostatically reduce this response following extended periods of sleep loss and that this reduction leads to behavioral consequences including disrupted sleep homeostasis and a reduction in sensitivity to hypnotic doses of alcohol. Over short time scales, we show that acute elevation of astrocytic calcium through InsP3R dependent liberation of this ion from intracellular stores produces an increase in astrocyte-derived adenosine. Finally, we demonstrate that the adenosine controlled by this pathway modifies synaptic transmission leading first to enhancement of synaptic plasticity when stimulated at low levels and then to presynaptic inhibition following more intense stimulation. These results strongly implicate astrocyte-derived adenosine in the effect of

wakefulness and sleep deprivation on hippocampal function and begin to paint a picture of the important but complex role of these cells in controlling synaptic and network function in the CNS.

Chapter 2: General Materials and Methods

Section 2.1: Overview

Studying astrocytic gliotransmission in a physiologically relevant context presents several major technical issues. Among the most significant of these challenges is the need to distinguish between the effects of transmitter molecules released by astrocytes and those released by neurons. To overcome this difficulty I have made use of two transgenic mouse lines, dominant negative SNARE (dnSNARE) and venus-tagged IP_3 Phosphatase (VIPP), which have deficits in SNARE dependent vesicular release and InSP3R dependent calcium signaling respectively. Because transgene expression in both of these mouse models is both conditional and astrocyte specific, they provide the means to isolate the effects of behavioral and pharmacological manipulations on these pathways and determine to what extent they contribute to adenosine signaling and its downstream effects on synaptic transmission.

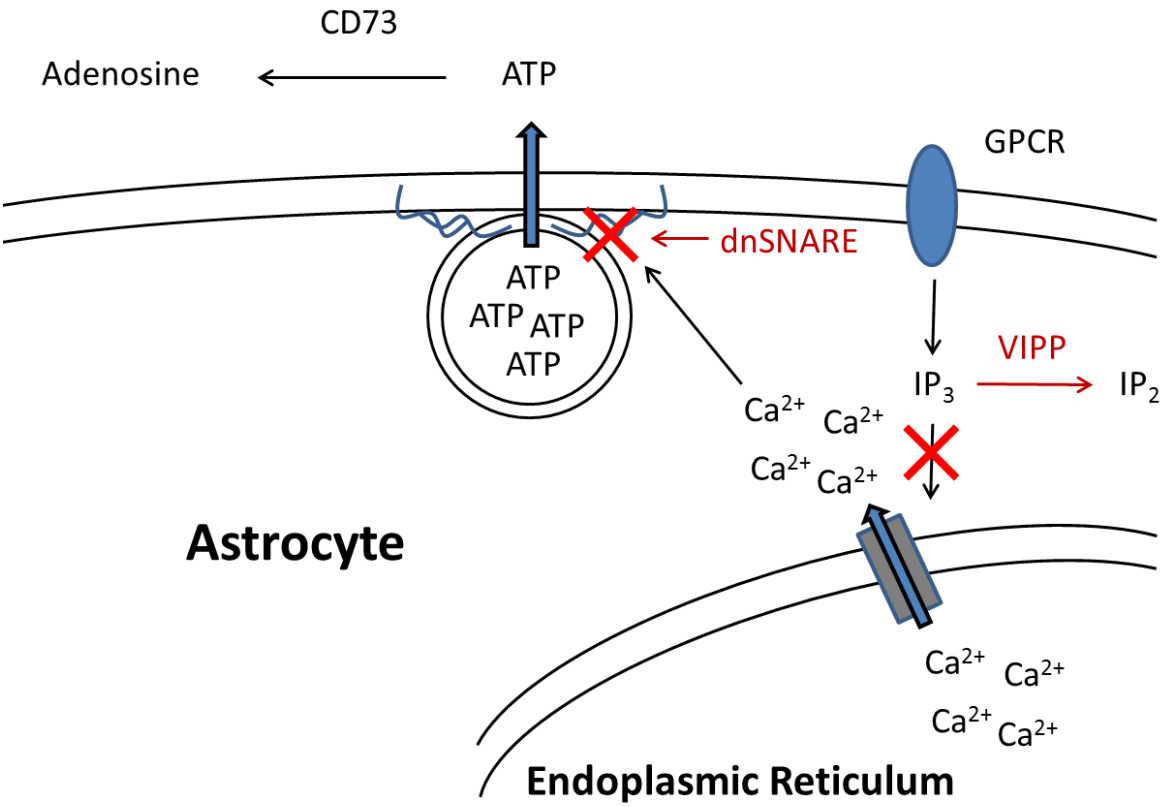
To measure the effects of wakefulness on astrocytic exocytotic signaling through adenosine, I have employed a variety of electrophysiological and electrochemical approaches both *in vivo* and in hippocampal slices. These include the following techniques: 1) Recordings of AMPA or NMDA receptor mediated field extracellular postsynaptic potentials (fEPSPs) *in situ* 2) Local field potential (LFP) based measurement of cortical activity in anesthetized mice and 3) Direct electrochemical measurement of adenosine and some of its metabolite using enzyme assisted biosensor based amperometry. In collaboration with Jerome Clasadonte I have also incorporated data from electroencephalographic (EEG) based measurements of behavioral state and

network activity to investigate the effect of long sleep disruption. Behavioral assays of alcohol sensitivity and blood alcohol content included in this publication were performed by Sally McIver while calcium imaging experiments in the hippocampal slice were completed by Dustin Hines.

Section 2.2: Derivation Care and Use of Transgenic Mouse Lines

Transgenic Mouse Models: All animals used for the research presented in this dissertation were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Tufts University Institutional Animal Care and Use Committee (IACUC). Mice (Mus Musculus) were used as a model species in all experiments. In the dnSNARE mouse line, a dominant negative fragment made up of the cytosolic portion containing amino acids 1 through 96 of the SNARE protein Vesicular Associated Membrane Protein 2 (VAMP2 also called Synaptobrevin 2) driven by the tet operator (tetO) was inserted randomly into the genome of one of the transgenic founder lines. In addition the gene encoding enhanced green fluorescent protein (eGFP) driven by the same promoter was inserted at a different locus to provide a marker for expression. The VIPP transgene is a tetO regulated gene encoding a fusion protein consisting of the venus yellow fluorescent protein (YFP) linked with the enzyme IP_3 phosphatase. Following insertion of the gene and derivation of a true breeding line the founders were backcrossed for at least 10 generations onto the C57 black 6 (C57bl6) background strain before use. Figure 2.1 shows the theoretical effect of these manipulations on astrocytic release.

Figure 2.1: Astrocyte Specific Transgene Expression: dnSNARE and VIPP



To achieve conditional, astrocyte specific expression, single transgenic mice from each of these lines were crossed with mice expressing the "tet-Off" tetracycline trans-activator (tTA) under control of the promoter for the astrocyte specific gene glial fibrillary acidic protein (GFAP). Astrocyte specific expression in the bigenic offspring of this cross was subsequently verified using fluorescence imaging for both eGFP (Pascual et al., 2005; Halassa et al., 2009b) and the VIPP fusion protein (Halassa and Lee unpublished observations). All mice were maintained on a diet containing doxycycline (Bio-serv) until weaning to suppress transgene express and prevent developmental effects of dnSNARE or VIPP. Control experiments were performed on littermates in which only one transgene in the system is present based on polymerase chain reaction (PCR) based genotyping. Mice were maintained on a twelve hour light/dark cycle for at least two weeks prior to each experiment to allow synchronization of their activity patterns with this circadian cycle.

Section 2.3: Experimental Methods

Sleep Deprivation: We used multiple methods to disrupt sleep in mice. In some experiments we used the well characterized method of gentle handling to sleep deprive mice. Previous studies have demonstrated that this approach effectively reduces sleep time in mice, leading to subsequent rebound in time and intensity of recovery sleep (Halassa et al., 2009b). In this method mice are continuously monitored by the experimenter over the course of the four to six hour (4-6h) sleep deprivation period. If the mice assumed a sleep posture at any point during this period, the bedding was gently disturbed and redistributed to wake the mouse. If this failed to awaken the mouse, a

nitrile laboratory glove was used to gently brush the mouse until it awoke. Mice were not habituated to handling prior to deprivation to reduce stress effects (Longordo et al., 2011).

In some cases mice were sleep deprived using an automated system based on gently enforced movement on a slowly moving treadmill. In these experiments, mice were placed in individual plexiglass chambers (46 sq inches X 1' 1" high) on top of a treadmill housed in a soundproof chamber. In all cases, mice were allowed approximately 1 week to acclimate prior to the start of sleep deprivation or sleep restriction (below). Food and water were available ad libitum in hoppers attached to the sides of the chambers throughout the habituation and deprivation period.

Chronic sleep restriction: Chronic sleep disruption was performed using the treadmill based sleep deprivation system as described above. The sleep restriction period began at the onset of the light cycle (Zeitgeiber time 0; ZT0, subjective nightime), and was implemented using 6 h cycles consisting of 4 h of treadmill on (speed of 3.33 cm/s) and 2 h of treadmill off, 4 times per day for 3 days. Undisturbed mice (non-sleep restricted controls) housed in the same soundproof chamber were used as controls. The parameters used in this protocol were based on a previous report using intermittent treadmill activity to restrict sleep (Bjorness et al., 2009).

Acute Hippocampal Slice Preparation: Horizontal slices were obtained from 8-12 week old mice following brief anesthesia using isofluorane. The brain was rapidly extracted in cold cutting solution (124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10

mM Glucose,1 mM Sodium Pyruvate, 2.9 mM KCl, 1mM $CaCl_2$, 2 mM $MgCl_2$) bubbled continuously with a 95% O_2 5% CO_2 gas mixture. Either 310 µm (for field recordings) or 400 µm (for biosensor recordings) thick slices were cut using a vibratome (VT 1200S; Leica). In slices used for biosensor based recordings, 0.6 mM Ascorbate was included in the cutting solution however this chemical was not included in slices used for biosensor recordings to prevent artifactual activation of the enzymes included in the sensors. For calcium imaging experiments, cutting solution included frozen sucrose added as needed to chill the solution. Following the cutting procedure, hippocampal slices were transferred to a bath containing continuously oxygenated cutting/recovery solution and incubated at 30˚ C for 1-1.5 hours prior to recording. In all cases, the zeitgeber time corresponds to the time of sacrifice. Mice were briefly exposed to light following anesthetization in order to perform the dissection.

Surgical Procedures: All acute, *in vivo* recordings as well as survival surgeries for EEG recordings and chronic pharmacology were performed by trained researchers using appropriate anesthetic agents to minimize pain and distress during and after each procedure. Throughout these surgical interventions, depth of anesthesia was assured by continuously monitoring respiration rate, eyelid reflex, vibrissae movements, and testing reactions to tail and toe pinching. Sterile technique was employed for all survival surgeries. During surgery exposed tissue was kept clean and hydrated with osmotically balanced saline solutions.

Targetting of electrodes for EEG and LFP recordngs as well as canulae for intracerebroventricular (i.c.v.) was based on stereotactic coordinates relative to the

lambda and bregma intersections of the coronal, sagittal and lambdoid sutures. Following implantation for chronic recordings, electrodes and headstage plugs were immobilized with minimal quantities of acrylic dental cement (vendor) and the scalp was sutured around the implant. During recovery, mice were monitored for pain and distress and analgesic quantities of Buprenorphine were supplied (vendor) as needed. After surgeries, mice were allowed at least one week recovery before recordings or behavioral manipulations.

Adenosine Biosensor Recording: Biosensor electrodes (Sarissa Biomedical Ltd, Coventry, UK) coated with an enzymatic matrix surrounding a platinum electrode (50 µm diameter) were was polarized to + 500 mV. Electrochemical detection occurred via amperometric measurement of hydrogen peroxide produced by the degradation reaction mediated by the enzymes included in the matrix (Frenguelli et al., 2003; Llaudet et al., 2003). To control for electrical noise and non-specific electrochemical signal, two sets of biosensor were employed. Adenosine biosensors (ADO) were coated in an enzymatic layer containing nucleoside phosphorylase, xanthine oxidase and adenosine deaminase while Inosine (INO) biosensors lacked adenosine deaminase and were therefore insensitive to adenosine. Before use, all electrodes were hydrated and precalibrated with 10 uM adenosine in aCSF (124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM Glucose,1 mM, 2.9 mM KCl, 2mM $CaCl_2$, 1 mM $MgCl_2$).

Using these electrode biosensors, adenosine levels were measured in situ in horizontal hippocampal slices. The ADO and INO sensors were inserted sequentially after which the slice was allowed a stabilization period of at least 20 minutes to avoid

aberrant signal produced by acute damage following sensor insertion. Adenosine sensors were always inserted first and removed last. This allows the difference in the signal between times when both sensors are present and others when only a single sensor is present to be used to estimate the metabolite component of the signal generated by inosine, hypoxanthine and urate. Post calibration with 10 µM adenosine standard was used to scale adenosine signal. Inosine at 10 µM was also applied at the end of experiment to calibrate the relative sensitivity of INO and ADO biosensors to inosine. Estimation of tonic extracellular adenosine was made using INO subtracted ADO signals following the 20 minute stabilization period. The value was scaled to the post-calibration standard in order to estimate concentration. Analysis used raw signal with no preprocessing unless otherwise stated.

Potentiostat based recordings were made using the ME200+ Duo-Stat (Sycopel International Ltd. Jarrow, UK) and were continually digitized via a Digidata 1320 digitzer at 10 kHz sampling rate (Molecular Devices). Acquisition and analysis utilized Clampex 9.2 software (Molecular Devices). During recording slices were perfused with aCSF at a rate of 1.2 ml/min with temperature maintained at 32.5-33 ºC.

Statistical Analysis: Following data acquisition and preliminary analysis using clampfit (axon instruments), SleepSign for Animal software (Kissei Comtec) or MATLAB® computing software (MathWorks, Natick MA) data was stored in spreadsheets created using Microsoft Excel. For all graphical visualizations distribution means, error bars correspond to standard errors of the mean (SEM) and n values correspond to the number of animals within each group. Statistical comparisons were performed in SigmaPlot 11

Software. Comparisons involving multiple categories or groups were initially analyzed with appropriate parametric or non-parametric analysis of variance (ANOVA) based on pre-testing of the distribution to determine normality. Allowed individual comparisons were made using Tukey or Student Neuman-Keuls t (SNK t-test) post-hoc tests. Normalized measures were compared using Mann-Whitney U test with Bonferroni correction or non-parametric ANOVA to account for multiple testing. For all comparisons, normality tests and equal variance tests were performed to ensure distribution properties were consistent with the tests used. In cases where data assumed to be normal failed the normality tests, non-parametric comparisons were substituted for the parametric comparison.

Chapter 3: Wakefulness Affects Synaptic and Network Activity by Increasing Extracellular, Astrocyte-Derived Adenosine

Contributing Authors: Sims, R. Dale, N. and Blutstein, T. were consulted during design of the research presented in this chapter.

Section 3.1: Overview

Short term sleep leads to an increase in sleep drive and deficits in hippocampal dependent memory within both humans and rodents. Both of these responses are thought to require activation of adenosine A1 receptors as well as SNARE dependent release of gliotransmitters from astrocytes since disrupting either of these signaling pathways results in comparable reductions in homeostatic sleep drive and improvements in memory consolidation with sleep loss (Halassa et al., 2009b; Florian et al., 2011). Because astrocytes are capable of providing a source of extracellular adenosine through exocytotic release of its precursor, ATP, these findings clearly imply that astrocyte-derived adenosine should increase in response to wakefulness. Moreover, the involvement of astrocytic gliotransmission in the effects of sleep loss on synaptic plasticity in isolated preparation (Florian et al., 2011), suggests that astrocyte-derived adenosine could occur locally in areas relevant to episodic memory such as the hippocampus. Although wakefulness-dependent changes in the concentration of extracellular adenosine have been observed in some brain regions (Kalinchuk et al., 2011) no previous study has determined whether these changes require release from astrocytes. In addition, despite several attempts to measure adenosine dynamics in the hippocampus using microdyalisis it remains unclear whether wakefulness-dependent changes in adenosine release or clearance occur in this structure.

To address these questions I have used multiple methods to assess the level of adenosine in the extracellular space following normal and enforced wakefulness and to determine the extent to which this adenosine modulates synaptic transmission. In addition, I have used molecular genetic inactivation of astrocytic exocytosis to determine whether changes in adenosine are mediated by vesicular release from this cell type. Finally, using direct measurement of adenosine I determined whether the rate of clearance of adenosine varied diurnally and whether such variation depends on phosphorylation by ADK. My results show that the level of adorA1R activation increases in response to wakefulness in mice (M. musculus). I found that this increase affected synaptic transmission in the hippocampus and modulated network activity in the cortex. Direct, biosensor-based measurement of adenosine showed that the net extracellular concentration of this transmitter increased in response to normal wakefulness and sleep deprivation. Genetic inhibition of gliotransmission prevented this increase and attenuated the wakefulness-dependent changes in synaptic and network regulation by adorA1R. Although clearance of exogenously applied adenosine does vary over the course of the light dark cycle due changes in ADK expression, this variation follows a trend opposite to the observed change in tonic adenosine levels. Consequently, I conclude that wakefulness increases the level of extracellular adenosine in the hippocampus and that this increase requires the release of transmitters from astroctyes.

Section 3.2: Specific Methods

Extracellular recordings: All fEPSP recordings were made in recording artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM

Glucose, 1 mM Sodium Pyruvate, 2.9 mM KCl, 2mM $CaCl_2$, 1 mM $MgCl_2$) continuously superfused at 1.2 mL/minute and maintained at 32.8 °C. Glass recording electrodes filled with aCSF were positioned in area CA1. Signals were amplified and band pass filtered between 0.1Hz and 1 kHz using a 1800 microelectrode amplifier (A-M Systems) and digitized at 5 kHz via a Digidata 1320 digitzer (Molecular Devices). The CA1-CA2 boundary was severed and paired stimulation pulses were delivered using a 125 µm concentric Pt-Ir electrode in the stratum radiatum to orthodromically stimulate axons entering area CA1 (0.033 Hz). Recordings were acquired with Clampex 9.2 software and analyzed using Clampfit (Molecular Devices).

Estimation of Tonic Adenosine A1 Receptor Mediated Inhibition (adenosine tone): The slope of field extracellular postsynaptic potential (fEPSP) was measured online and stimulus intensity/response curves were obtained by supplying stimulating currents of increasing intensity between 50 and 175 µA to estimate the maximum slope at saturation. Paired pulse recordings were made throughout the experiment with an interstimulus interval of 25 ms between pulses within each sweep. Following a 30 minute baseline recording period, inhibitory tone mediated by AdorA1R was estimated by perfusing the slice with the AdorA1R antagonist 8-Cyclopentyl-1,3-dimethylxanthine (CPT; 200nM). The increase in the slope of the field potential following CPT application was used to estimate the degree of tonic inhibition (adenosine tone) according to the following equation (Adenosine Tone = ([fEPSP.cpt – fEPSP.acsf] / fEPSP.cpt)).

In Vivo Extracellular Recordings: Adult mice aged between 6–14 weeks were anesthetized by an intraperitoneal injection of urethane (2 g/kg) in two doses over a one hour period. Body temperature was measured with a rectal probe and kept at 37 °C with a heating pad. To maintain head position a metal plate was adhered with cyanoacrylate glue onto the skull and affixed to a stable platform. A craniotomy (1.8 mm) was then drilled in the skull overlaying the somatosensory cortex. The surface of the cortex was kept moist with normal HEPES-buffered artificial cerebrospinal fluid (125 mM NaCl, 5 mM KCl, 5 mM Glutamate, 10 mM HEPES, 3.1 mM $CaCl_2$, 1.3 mM $MgCl_2$) titrated to pH 7.4 using 1M NaOH. The dura was then carefully dissected to expose the cranial surface. Local field potentials (LFPs) were recorded with custom-built electrodes made of two parallel tungsten electrodes (FHC) which were positioned to record from the superficial layers of the somatosensory cortex. Signals were amplified with an AM-amplifier (AM-system), filtered at 0.1 Hz-10 KHz, and digitized at 50 KHz. Recordings for baseline were started at least 10 min after the electrodes were inserted in the cortex to allow signal stabilization. Recordings were acquired using Clampex 9.2 software (molecular devices).

Analysis of in Vivo Extracellular Recordings: For LFP analysis data were sampled at 10 KHz and low-pass filtered at 100 Hz. Power spectra were obtained by averaging a rectangular window over a time period of either 5–10 min (for cumulative effects after 20 minutes) or 27s (for time dependent evolution analysis). Power spectra after a given compound was applied were calculated 20 min after drug application to the surface of the cortex for comparison of overall effects. Power spectra were normalized by the average

power at each frequency in the baseline recording period prior to drug application. Slow oscillation power was calculated by integrating the power spectrum between 0.36 to 1.09 Hz (slow oscillation range, 0.4–1 Hz). To determine whether slow oscillations showed time dependent evolution in the absence of CPT, I tested whether the baseline slow oscillation power was correlated with time using Spearman's rank correlation test of the normalized baseline. I observed that a subset of recordings in each group showed apparent baseline instability and an upward drift in slow oscillations power (1-2 per group, 6 of 24 total). To avoid potential bias in evaluating the effect of CPT, I did not retain these values in subsequent analysis. Analysis was performed using Clampfit (Molecular Devices) and SigmaPlot software (Systat).

Adenosine Clearance Assay: Biosensor based recordings were performed as described previously (Chapter 2). In order to determine the whether there is diurnal variation in the rate of clearance of adenosine, a fixed concentration of adenosine was superfused onto acute hippocampal slices taken following either the normal wakefulness period at ZT 0 or following the normal period of sleep at ZT 12. Recordings were made as described above. The ADO and INO biosensors were then positioned in the central portion of the slice to measure the relative concentration reaching this depth. Following the initial recovery period after insertion, 2 or 10 uM adenosine was bath applied for 10 minutes. This adenosine was then washed out for at least 10 minutes prior to either a second application of adenosine or removal of the biosensor from the tissue. Calibration was then conducted as previously described using 10 uM adenosine and inosine. In some

experiments the ADK inhibitor Iodotubericidin (IODO) was applied at a concentration of 4 uM.

Biosensor Signal Analysis: As the sensitivity of both INO and ADO electrodes are linearly proportional to inosine over a relatively broad range (100 nM- 100 uM) and the inosine biosensor is insensitive to adenosine (Dale et al 2000; Frenguelli et al, 2003), I corrected the raw signals to account for different sensitivities of each pair of electrodes. To do this I adjusted the inosine signal using the ratio of the sensitivity of the ADO and INO electrodes to the inosine standard. The resulting INO response was then subtracted from the ADO signal to obtain the current response specific to adenosine. Finally, this signal is calibrated to the adenosine standard to provide the concentration estimate. The capacitative drift in the raw signals was determined based on a first order exponential fit of the signal measured in the slice chamber prior to placement of the electrodes and was subtracted before scaling. Signal analysis was performed using SigmaPlot (Systat) and MATLAB ® computing software (MathWorks, Natick MA)

Section 3.3: Results

Increased activation of adenosine A1 receptors following normal wakefulness

To determine the impact of wakefulness-dependent adenosine signaling on hippocampal synaptic transmission I initially assessed the baseline synaptic properties in hippocampal slices taken from mice at different time points in their normal sleep/wake cycle. I recorded fEPSPs in slices taken from mice sacrificed at four hour intervals during the light dark cycle. Zeitgeber time 0 (ZT = 0) corresponds to the onset of the light phase

Figure 3.1:

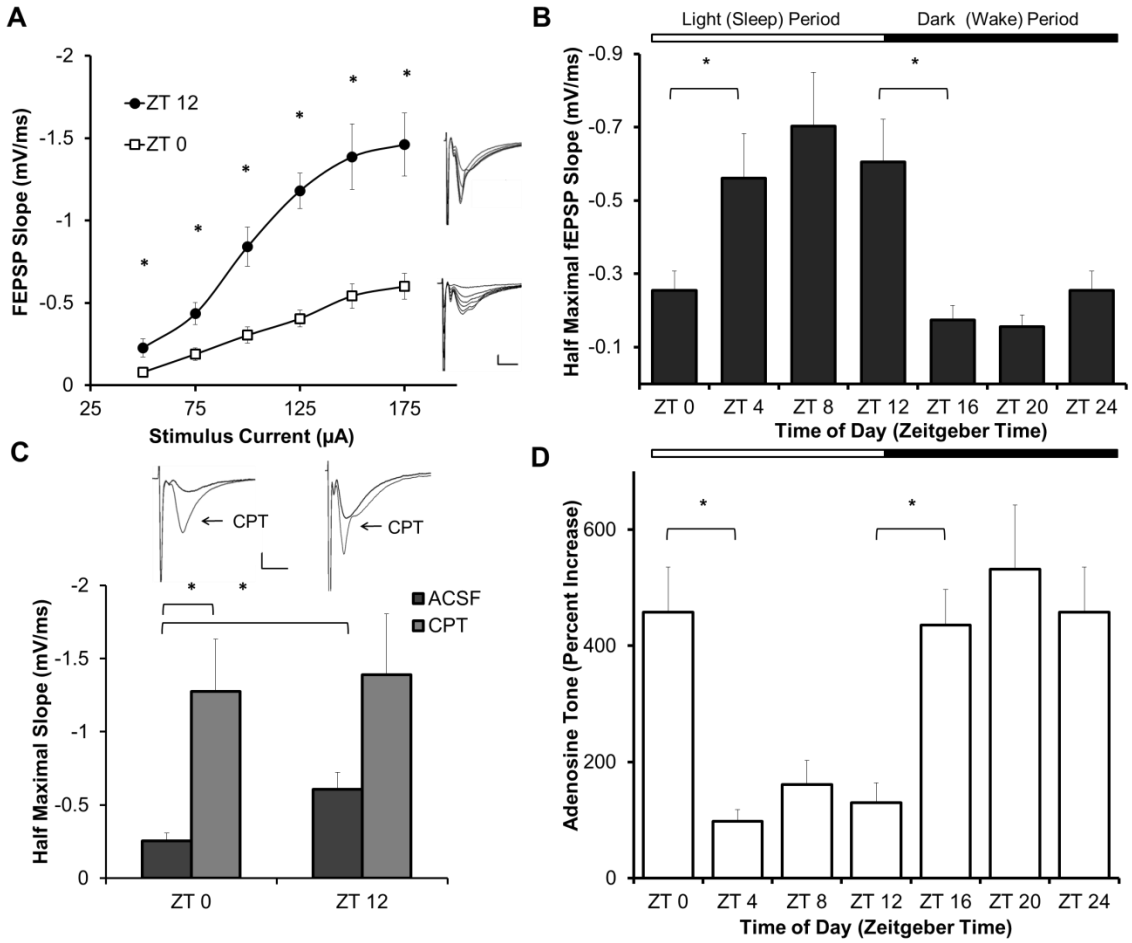


Figure 3.1: Diurnal changes in adorA1 receptor mediated inhibition of excitatory synaptic transmission. (A) fEPSPs slopes were significantly greater in slices taken following the normal sleep period (ZT 12) compared to slices taken following wakefulness (ZT 0) (two way, repeated measures ANOVA, post-hoc SNK t-test: * $p < 0.05$, $n = 12$). Inset: typical fEPSP traces recorded at increasing stimulus currents (scale bar 0.5 mV, 5 ms). (B) Slices obtained during the dark phase showed consistently lower half maximal fEPSP slopes relative to slices taken during the light phase (ANOVA, $p < 0.005$, post-hoc SNK t-test: * $p < 0.05$, $n = 6$). (C) The adorA1R antagonist CPT eliminated differences in fEPSP from slices obtained at ZT = 0 and ZT = 12 ($p < 0.001$, ANOVA, Tukey test: * $p < 0.05$, $n = 6$). Inset: typical half maximal fEPSP traces before (black) or following (red) application of CPT (scale bar 0.5 mV, 5 ms). (D) Tonic adorA1 receptor-mediated inhibition was significantly greater in slices taken in the dark compared to the light phase ($p < 0.001$, Kruskal-Wallis non-parametric ANOVA, Tukey test: * $p < 0.05$, $n = 6$).

(subjective night time) and ZT 12 to the onset of the dark phase (subjective daytime). Input-output curves elicited in stratum radiatum of area CA1 revealed that the magnitude of the fEPSP was greater at the end of the light phase (ZT = 12) compared to the end of the dark phase (ZT = 0; Fig. 3.1A). Comparison of half-maximal fEPSP slope recordings showed consistently reduced responses during the dark phase which transitioned to a higher response slope after the first four hours of the light phase (Fig. 3.1B). No difference was observed for half-maximal presynaptic volley amplitude in slices taken at ZT = 0 (0.27 ± 0.03 mV) compared with those taken at ZT = 12 (0.32 ± 0.06, $p = 0.46$ SNK t-test, n = 6) suggesting that changes in axonal conduction do not contribute to the change in fEPSP slope. The paired pulse ratio of fEPSP slope in slices taken at the end of the dark phase (ZT = 0; 2.08 ± 0.15) was significantly greater than that obtained from slices harvested at the end of the light phase (ZT = 12; 1.53 ± 0.15, $p < 0.05$ Mann-Whitney U test) consistent with the idea that the change in fEPSP slope was caused by an increase in presynaptic inhibition.

Because net extracellular adenosine has been shown to rise in the dark phase during wakefulness in the cortex and BF (Kalinchuk et al., 2011), and because adenosine is known to cause a presynaptic adorA1 receptor dependent inhibition of synaptic transmission (Cunha et al., 1998), I determined whether the level of adorA1 receptor activation in hippocampal slices was changed as a function of the sleep/wake cycle. To isolate the pool of tonic extracellular adenosine acting on hippocampal adorA1 receptors, I used an in situ adenosine tone assay in acutely harvested slices. Inhibitory adenosine tone was estimated by applying CPT (200 nM), an adorA1R specific antagonist, and measuring the increase in slope of fEPSP. Slices were harvested from mice sacrificed at

four hour intervals across the light-dark cycle starting at the onset of sleep (ZT = 0). Consistent with the previously measured input-output curves, the half maximal fEPSP slope was significantly lower in slices harvested following the normal period of activity (ZT = 0) compared with slices taken following the normal sleep period (ZT = 12) however this difference was absent following application of CPT (Fig. 3.1C). Application of CPT also significantly reduced the paired pulse ratios in both groups of slices (ZT 0: 2.08 ± 0.15 to 1.30 ± 0.15 in CPT, ZT 12: 1.53 ± 0.15 to 1.13 ± 0.07 in CPT). There was no significant difference between the paired pulse facilitation following application of CPT ($p = 0.59$, Mann-Whitney U test) between slices obtained at ZT =0 and ZT = 12 further suggesting that adorA1 receptor activation is responsible for the increased presynaptic inhibition observed in ZT 0 slices. The magnitude of basal synaptic transmission was inversely related to the level of adenosine tone which showed a strong negative correlation with fEPSP slope (Correlation Coefficient = -0.6, $p < 0.0005$ Spearman Rank Order Correlation). Adenosine tone was consistently higher in slices taken during or directly following the dark phase and was significantly lower in slices taken following the first four hours of the sleep period (Fig. 3.1D). These results suggest that tonic activation of adorA1 receptors is increased during the dark phase (subjective daytime) compared to the light phase (subjective nighttime). An interesting aspect of these findings is the apparent stability of the level of tonic adenosine during the light phase and relatively rapid transition between the light and dark phases. Although the precise time-course is unclear the change within a four hour period is faster than what has typically been observed using microdialysis based methods in other brain regions (Kalinchuk et al., 2010). This may be due to the differential sensitivity of the techniques

since changes in synaptic adenosine, which are measured in the adenosine tone assay, are not accessible in microdialysis based measurements. It may also be that increased extracellular adenosine is triggered by signals tightly associated to transitions to wakefulness such as diffuse modulatory signals from the ascending arousal pathway. Determining the precise mechanism by which this apparent "switch" occurs will require additional investigation.

One potential concern with the use of *in situ* measurement of tonic adenosine is that adenosine accumulated during periods of wakefulness could be diluted during the recovery period and the effect of wakefulness might therefore be disrupted by incubation. To address this concern we determined whether a lower adenosine tone was observed following longer periods of incubation. Across all recordings, we found that neither adenosine tone (Correlation Coefficient = 0.206, p = 0.72 Spearman Rank Order Correlation) nor fEPSP slope (Correlation Coefficient = -0.05 p = 0.67; Pearson Correlation) was correlated with the length of incubation (1-2 hours, average incubation = 97 minutes). This observation suggests that adenosine tone is preserved at a consistent equilibrium between release and clearance following incubation over the range of time used in this study.

Gliotransmission is Necessary for Wakefulness-Dependent Increase in adorA1 Receptor Activation

To determine whether the diurnal modulation of fEPSP magnitude and adenosine tone is regulated by wakefulness, I subjected mice to a period of sleep deprivation (induced by gentle handling) between ZT = 0 and 4 prior to cutting hippocampal slices.

Figure 3.2:

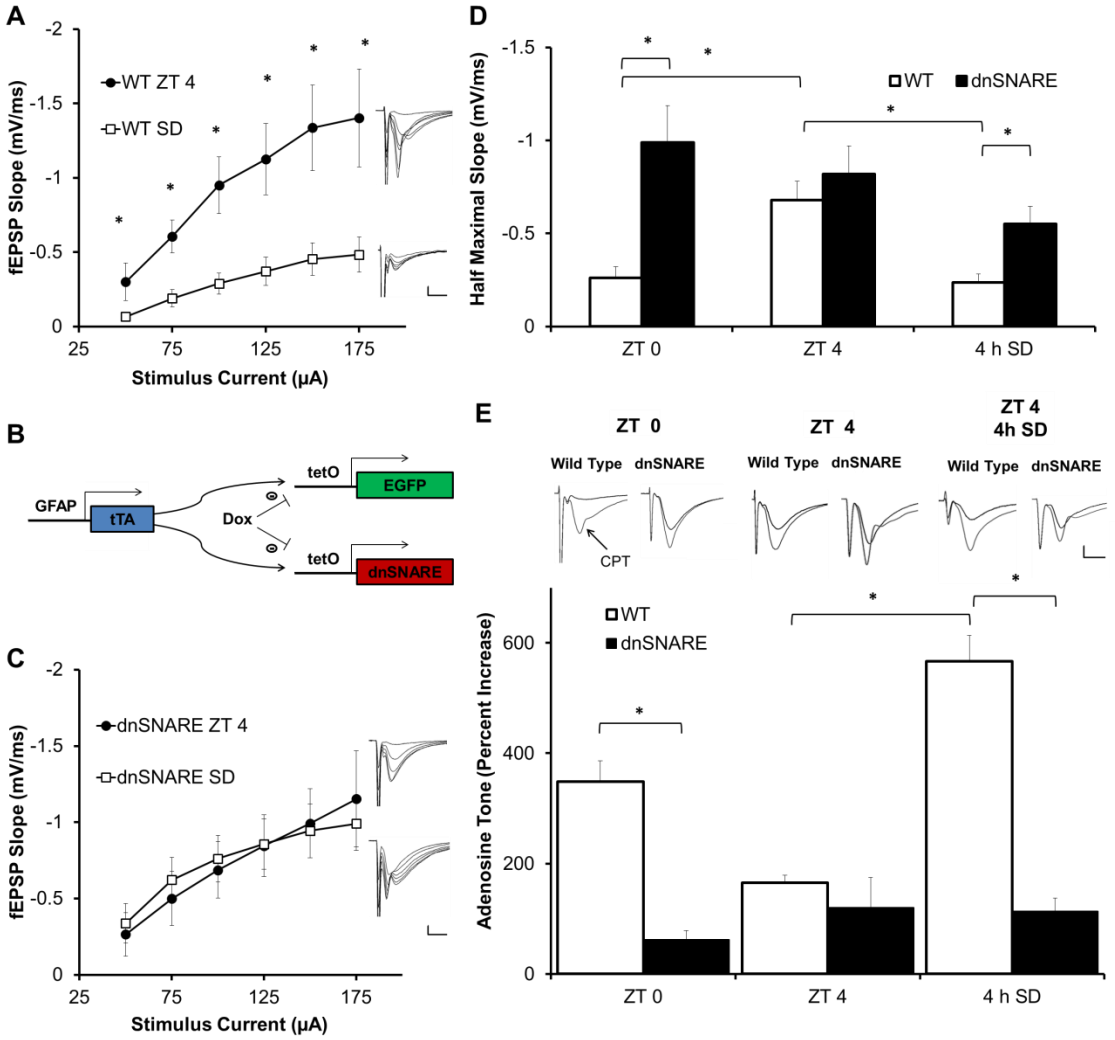


Figure 3.2: Wakefulness-dependent modulation of adorA1 receptor-mediated presynaptic inhibition requires an astrocytic SNARE-sensitive pathway. (A) Four hours of sleep deprivation prior to slice preparation significantly reduced fEPSP slope in wild type (WT) mice ($p < 0.005$, two-way, repeated measures ANOVA, post-hoc SNK t-test: * $p < 0.05$, n = 6). (B) Schematic diagram depicting the dnSNARE mouse model which uses conditional, dominant negative suppression of SNARE function under the GFAP promoter to drive the expression of dnSNARE and EGFP (reporter) in astrocytes. (C) fEPSP slopes in slices from dnSNARE mice were not sensitive to sleep deprivation. Insets show fEPSP traces for increasing intensities of stimulation (scale bar 0.5 mV, 5 ms). (D) Half maximal fEPSP slope was significantly higher in dnSNARE slices taken following normal wakefulness (ZT = 0) and following four hours of sleep deprivation but not following four hours of undisturbed sleep ($p < 0.005$, two way ANOVA, post-hoc SNK t-test: *$p < 0.05$, n = 6). (E) Tonic inhibition by adenosine adorA1 receptors was significantly increased by sleep deprivation in slices taken from WT, but not dnSNARE, mice (Mann-Whitney U test (Bonferroni Correction): * $p < 0.05$ n = 6).

Input output curves revealed that sleep deprivation significantly reduced the fEPSP response at increasing stimulation currents in slices taken from WT mice (Fig. 3.2A). Previous studies have shown that astrocytes play an important role in the homeostatic response to sleep deprivation (Halassa et al., 2009b). The involvement of this cell type is thought to involve the release of ATP, which in the extracellular space is hydrolysed to adenosine (Dunwiddie et al., 1997), through a mechanism that depends on astrocytic SNARE function (Pascual et al., 2005). I therefore used a line of mice expressing a conditional, dominant negative transgene specifically in astrocytes to disrupt release of transmitters. The transgenic mouse model uses the tetracycline trans-activator from the "tet-off" system (Morozov, 2003) under the GFAP promoter which regulates the expression of "dnSNARE", the cytosolic portion of synaptobrevin II, to disrupt SNARE complex formation specifically in astrocytes (Fig. 3.2B). In contrast to results obtained in WT mice (Fig. 3.2A), the slope of the fEPSP in slices obtained from dnSNARE mice was insensitive to sleep deprivation (Fig. 3.2C). The presynaptic volley amplitude at half maximal stimulation was similar under both sleep deprived and undisturbed conditions in both genotypes ($p = 0.38$, two-way ANOVA, $n = 6$).

Because fEPSP slope in dnSNARE slices is insensitive to prior sleep deprivation I asked whether the fEPSP is similarly insensitive to diurnal modulation. While the slope of the fEPSP in WT mice significantly increases between ZT= 0 and ZT = 4 I found that there was no significant difference in fEPSP in dnSNARE slices at these two time points. Expression of dnSNARE significantly increases the fEPSP slope at ZT = 0 compared to that recorded from WT slices (Fig. 3.2D). The half maximal slope was significantly

Figure 3.3:

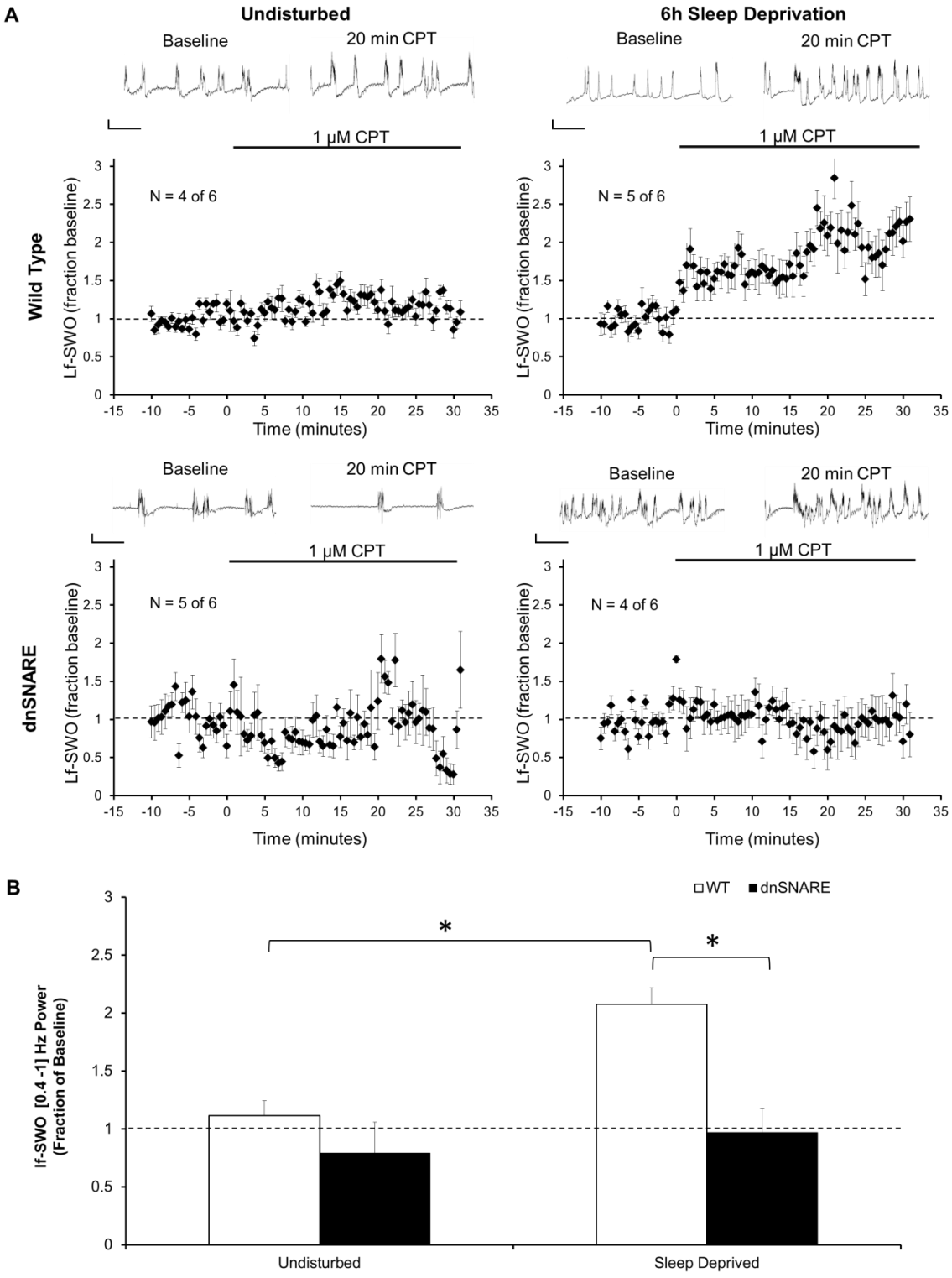


Figure 3.3: The effects of the adorA1 receptor antagonist CPT on cortical slow oscillations are potentiated by sleep loss through an astrocytic SNARE sensitive pathway.

(A-D) Local field potential (LFP) recordings were made in the somatosensory cortex of either WT or dnSNARE mice. Application of CPT produced an increase in power of slow oscillations (0.4 -1 Hz) in WT but not dnSNARE mice and this increase was potentiated by sleep deprivation (FFT of 27 second spectral segments, normalized to baseline). Inset traces show typical LFP recordings during baseline or following CPT (30 s recording at time = 22 minutes, scale bar: 2.5 mV, 5 s). (E)The average increase in slow oscillations following twenty minutes of CPT was significantly potentiated by sleep deprivation however the effect of CPT following sleep deprivation was significantly attenuated in dnSNARE mice (FFT average of 10 minute block; Mann-Whitney U test (Bonferroni Correction): * $p < 0.05$ n = 4-5).

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reduced in slices taken from WT but not dnSNARE mice following sleep deprivation, consistent with a wakefulness-dependent effect (Fig. 3.2D).

Since astrocytic dnSNARE expression is known to lead to reduced adorA1 receptor-dependent presynaptic inhibition of excitatory synapses, I asked whether effects of sleep deprivation were mediated through this glial dependent signaling pathway. Four hours of sleep deprivation significantly increased the adenosine tone measured in WT slices compared to that measured in WT slices obtained at the same ZT time from undisturbed controls (Fig. 3.2E). In contrast to WT, slices taken from dnSNARE mice did not exhibit significant time of day or wakefulness-dependent changes in adenosine tone: For example, adenosine tone was significantly reduced compared to WT slices both after normal wakefulness at ZT 0 and following sleep deprivation (Fig. 3.2E). Adenosine tone of WT animals was negatively correlated with fEPSP slope for WT (Correlation Coefficient = - 0.69, $p < 0.005$) suggesting that synaptic transmission is reduced by increased adorA1 activation. Together, these results suggest that wakefulness increases tonic adenosine mediated presynaptic inhibition of excitatory synaptic transmission through adorA1 receptors and that this effect requires an astrocytic dnSNARE sensitive pathway.

Wakefulness increases adorA1 receptor modulation of cortical network activity through an astrocytic SNARE-dependent mechanism

Measurement of adenosine tone in the hippocampal slice supports the hypothesis that wakefulness increases the level of adenosine mediated inhibition through a dnSNARE sensitive mechanism; however the interpretation of these findings is complicated by the use of an in situ preparation which may affect the levels of adenosine or adorA1R expression and cellular metabolism (zur Nedden et al., 2011). To overcome this potential issue, I next used an in vivo recording method to assess the level of modulation of spontaneous brain activity by adenosine following differing periods of prior wakefulness. I performed local field potential (LFP) recordings in the intact somatosensory cortex of dnSNARE and WT control mice and compared the effect of acute adorA1 receptor inhibition by topical application of 1 µM CPT to the cortical surface.

Initially, I measured the effect of CPT under baseline conditions in mice recorded six hours into their normal sleep period (ZT 6). Consistent with previous reports, under urethane anesthesia activity was characterized by synchronized bursts of activity during cortical "up" states interspersed in longer silent episodes or "down" states (Fig. 3.3A, insets). In order to obtain a non-biased estimate to quantitatively assess the level of adorA1 receptor mediated modulation, I performed fast Fourier transform (FFT) analysis of the raw signal and compared the resulting power spectrum under baseline conditions with the resulting spectra following application of CPT. The average period of peak

synchronized activity was 1-2 seconds giving a peak power in the slow oscillation range of approximately 0.4-1 Hz.

In agreement with measurements in situ (Fig. 3.2E), tonic adorA1R activation in vivo was low in undisturbed mice during the sleep period (ZT = 6). Addition of CPT to the cortex of undisturbed WT mice did not significantly change slow oscillation power (Fig. 3.3A). However, prior sleep deprivation potentiated the level of tonic adorA1 receptor activation since application of CPT in sleep deprived mice enhanced the power of slow oscillations (Fig. 3.3B). Application of CPT had no effect on slow oscillation power in dnSNARE mice under either undisturbed conditions (Fig. 3.3C) or following sleep deprivation (Fig. 3.3D). Following sleep deprivation, the effect of CPT on slow oscillations was significantly attenuated in dnSNARE mice compared to WT controls (Fig. 3.3E). These results demonstrate that extended wakefulness during sleep deprivation increases the modulation of cortical network activity by adorA1 receptors and that this increase requires normal SNARE complex formation within astrocytes. Importantly, these in vivo results corroborate our in situ observations by demonstrating that an astrocytic dnSNARE sensitive mechanism is required for forced wakefulness to increase the activation of adorA1 receptors.

Inhibition of SNARE-mediated gliotransmission attenuates wakefulness-dependent increase in basal extracellular adenosine in the hippocampus

To directly test the hypothesis that astrocytes are required for wakefulness-dependent regulation of extracellular adenosine I used enzymatic adenosine biosensors (Frenguelli et al., 2003; Llaudet et al., 2003). These microelectrode based adenosine

Figure 3.4:

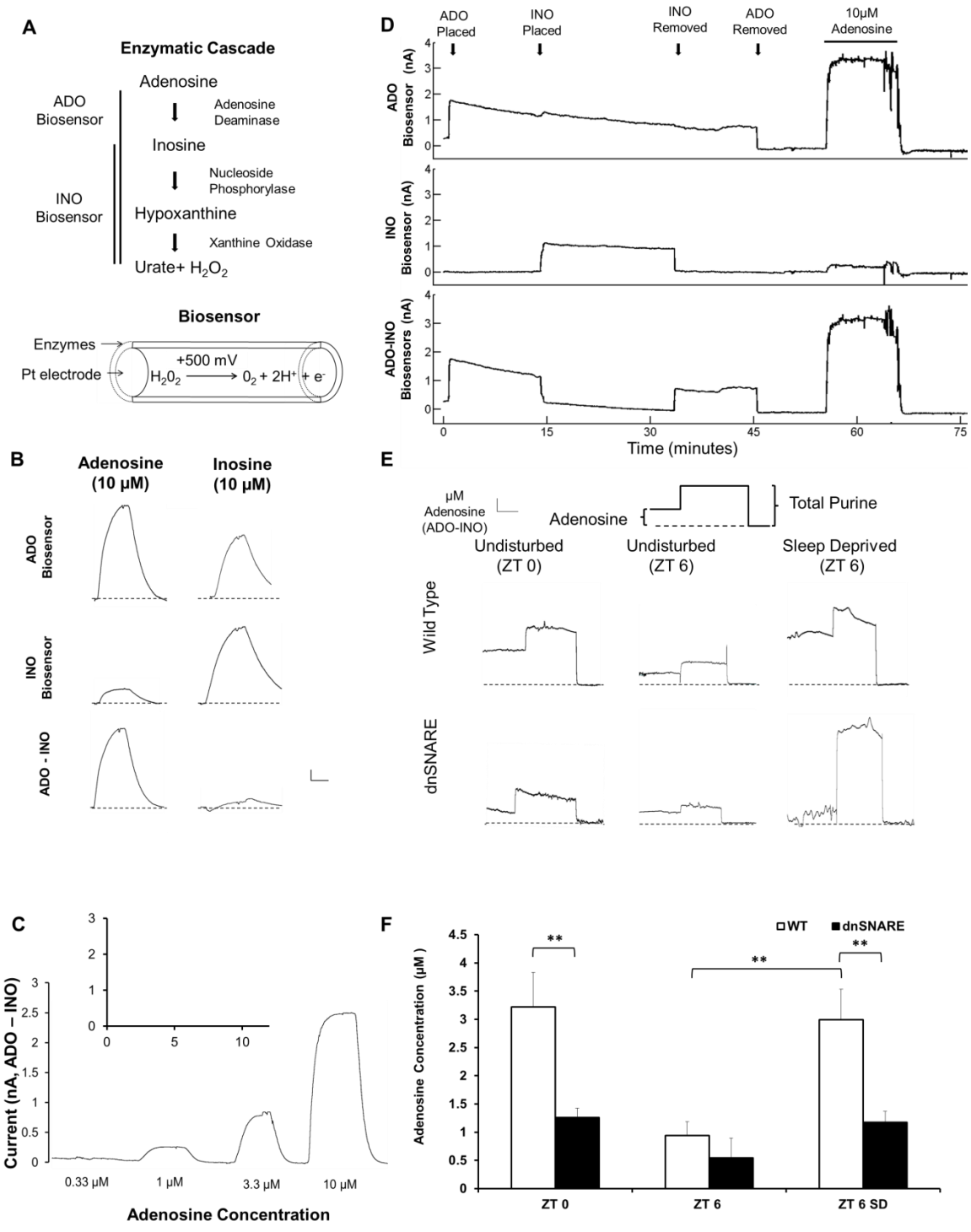

Figure 3.4: Wakefulness increases the level of hippocampal extracellular adenosine in situ through an astrocyte-dependent mechanism. (A) Enzymatic conversion of adenosine on the surface of biosensors produces peroxide species which are detected electrochemically. (B) Traces showing relative sensitivity of adenosine (ADO) and

inosine (INO) electrodes to 10 µM adenosine (left) and inosine (right) standards. Scaled subtraction of the INO signal was used to isolate the adenosine component from other intermediated products in the enzymatic cascade. (C) Calibration curve of the adenosine biosensor with inosine correction showing linearity over the 0.3-10 µM range. (D) Representative trace of adenosine measurement: ADO biosensors are lowered into area CA1 followed by INO biosensors. Following a 20 minute stabilization period, INO biosensors and then ADO biosensors were removed. (E) Typical sample ADO-INO traces in WT and dnSNARE slices taken following differing conditions of wakefulness. Inset diagram (top) shows the segments corresponding to the adenosine signal measured with and without INO control electrode insertion into hippocampal area CA1. Samples were scaled relative to post-calibration (Scale-bar: concentration scaled for dual electrode measurement of adenosine, µM; 5 minutes) (F) Adenosine was significantly increased in WT but not dnSNARE slices taken following sleep deprivation ($p < 0.005$ two way ANOVA, Tukey test: ** $p < 0.01$, $n = 6$).

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biosensors employ sequential enzymatic conversions to produce an electrochemical signal proportional to the concentration of adenosine (Fig. 3.4A, ADO biosensors) or its major metabolite in the extracellular space, inosine (Fig. 3.4A, INO biosensors). Because ADO biosensors are also sensitive to metabolites of adenosine, I used INO biosensors to isolate the adenosine specific signal. To compensate for possible differences in the relative sensitivities, I calibrated both sets of biosensors following each experiment with 10 µM adenosine and inosine standards and adjusted the differential signal based on the current response of each electrode (Fig. 3.4B). Application of increasing concentrations of adenosine demonstrated that the ADO biosensor responded linearly between 0.33 and 10 µM adenosine (Fig. 3.4C; $R > 0.99$). I sequentially positioned the ADO and INO biosensor in contact with the surface of the tissue until a slight deformation of its surface was observed, indicating that the electrode was embedded (Fig. 3.4D). Comparison of the resulting signal to adenosine and inosine standards allowed us to obtain a quantitative measure of the equilibrium concentration of extracellular adenosine in slices taken from mice under different conditions of prior wakefulness (Fig. 3.4E). In slices harvested

following normal wakefulness at ZT 0, the measured concentration of adenosine was significantly higher in WT relative to dnSNARE slices (Fig. 3.4E, F). To determine whether this increase was an effect of prior wakefulness, I sleep-deprived WT and dnSNARE mice for six hours before sacrifice and compared slices taken from these animals with others harvested from undisturbed controls. Sleep deprivation significantly increased the level of extracellular adenosine in slices from WT mice however this effect was attenuated in slices from dnSNARE mice (Fig. 3.4F). Following sleep deprivation, the concentration of adenosine measured in WT slices was comparable to the value measured at ZT 0 and was significantly increased relative to slices taken from sleep deprived dnSNARE mice (Fig. 3.4F). I also observed a trend toward increased levels of adenosine metabolites in sleep deprived dnSNARE mice (3.29 ± 0.74 μM) compared to WT (1.51 ± 0.56 μM) however this effect did not reach significance ($p = 0.17$, $n = 6$, SNK t-test). These findings support the hypothesis that wakefulness increases the level of extracellular adenosine within the hippocampus through an astrocytic SNARE dependent mechanism.

Adenosine clearance is increased following normal wakefulness through an ADK dependent mechanism.

Extracellular adenosine concentration is controlled both by the rate of delivery from active sources and the rate of clearance, primarily via passive reuptake into astrocytes. The observed difference in tonic adenosine observed following wakefulness may therefore involve a change in the rate of clearance of this molecule. To test this possibility, I measured the relative clearance of exogenous adenosine superfused onto

Figure 3.5:

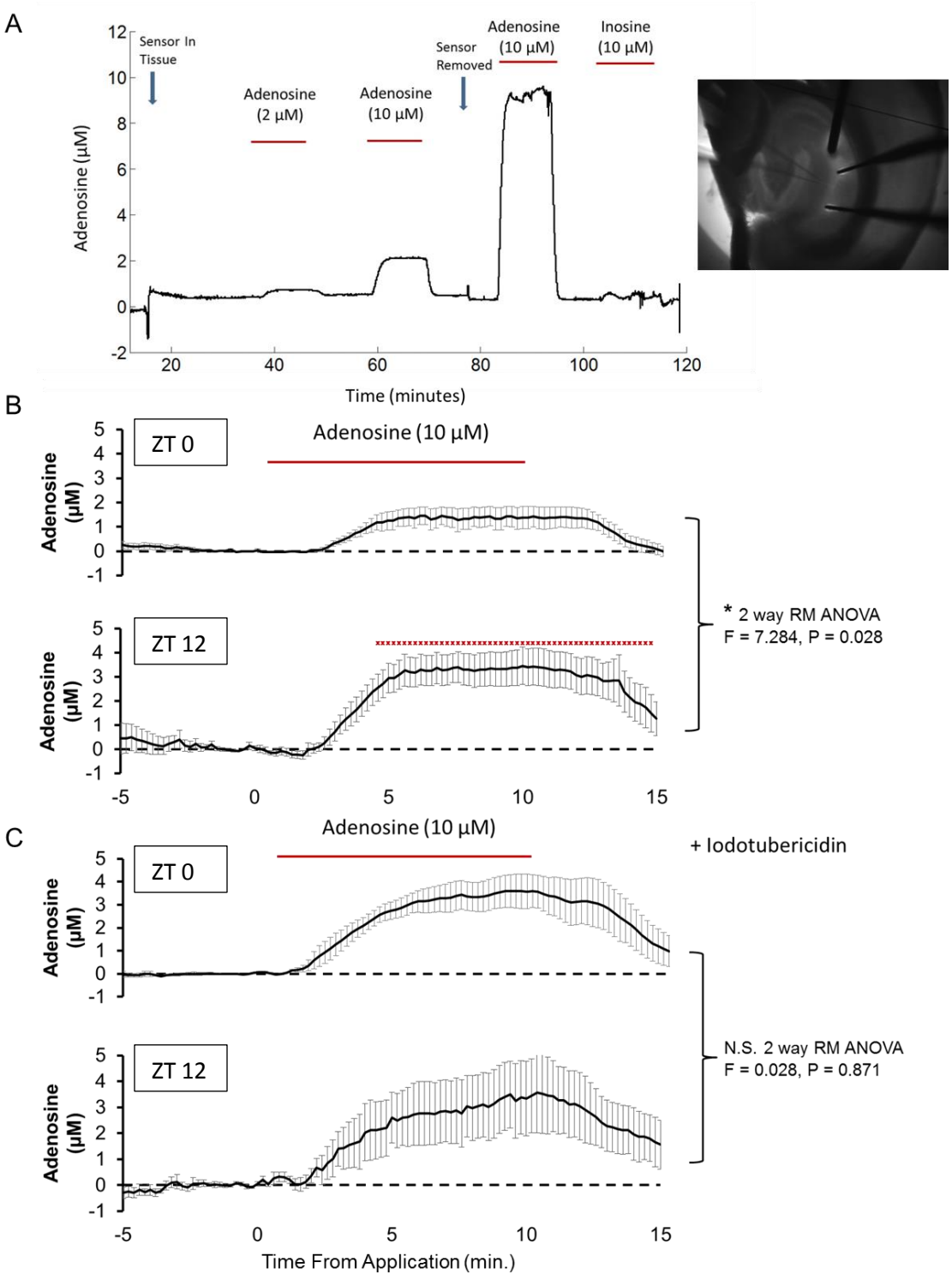

Figure 3.5: Adenosine clearance is enhanced following wakefulness through an ADK dependent mechanism. (A) Example trace taken at ZT 0 showing the adenosine signal obtained using the calibrated ADO/INO sensor responses. Inset: recording setup

showing positioning of each biosensors at the same focal plane within the hippocampal slice. Sensors were positioned so that the entire tip (50 um) was submerged within the slice. (B) Average response to 10 minute application of 10 uM adenosine. At saturation the concentration was significantly greater in slices taken at ZT 12 compared with that observed in slices taken at ZT 0 ($p < 0.05$ two way RM-ANOVA, Tukey test: * $p < 0.05$, $n = 5$). (C) The level of adenosine measured is not different in ZT 0 and ZT 12 slices in the presence of the ADK inhibitor Iodotubericidin (IODO, 4uM) ($p = 0.87$ two way RM-ANOVA).

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hippocampal slices. For this experiment, the sensors were inserted to approximately the center of the slice. Under equilibrium conditions, the concentration at a fixed depth within the slice depends on the rate of diffusion through the slice as well as the rate of clearance by enzymatic degradation and uptake into cells along the diffusion path (Dunwiddie et al., 1997; Wall et al., 2007). Thus, the concentration measured by the biosensor should be considerably lower when inserted into the tissue compared with the concentration measured when the sensors are removed from the slice. Consistent with this idea, in a single recording under constant conditions the calibrated signal in response to application of 10 uM adenosine was markedly reduced when the sensors were placed within the tissue compared with the signal following removal (Fig. 3.5A). To determine whether clearance of adenosine varies with time of day, the concentration observed at the center of the slice was compared between slices taken at either ZT 0, following normal wakefulness, or at ZT 12 following normal sleep. Assuming that the tortuosity of the tissue, which determines the diffusion rate, does not vary over the diurnal cycle, the concentration observed should be determined primarily by the effective clearance within the tissue. If tonic adenosine levels are controlled by the rate of clearance, one would expect that the higher adenosine tone observed at ZT 0 would correspond to a lower rate of clearance at this time point. Surprisingly, however, the response to exogenous

adenosine was significantly lower at this time point compared with the response in slices taken at ZT 12 (Fig. 3.5B), indicating that clearance is *higher* following wakefulness. This observation strongly argues against the hypothesis that wakefulness-dependent changes in adenosine are mediated by changes in the rate of removal from the extracellular space.

Adenosine clearance is thought to be mediated by two major mechanisms. First, adenosine can be metabolized to inosine by adenosine deaminase which is thought to be located primarily in the extracellular space (Yegutkin, 2008). Although higher expression of this enzyme could lead to higher rates of clearance, if this were the case a higher concentration of metabolites should be observed in response to exogenous application of adenosine in slices taken at ZT 0 relative those taken at ZT 12 which was not observed in these recordings (Time-Course Comparison: 2-Way RM ANOVA $F = 2.34$, $p = 0.17$). This argues against the interpretation that changes in adenosine deaminase are responsible for the more effective adenosine clearance following wakefulness.

Adenosine clearance can also be mediated by passive reuptake into neurons and astrocytes through ENT-1 channels. The net intracellular gradient that facilitates this clearance is thought to be maintained by efficient conversion of adenosine into AMP. This reaction is catalyzed by the kinase ADK, which is highly enriched in the cytosol of astrocytes. To test whether this enzyme was responsible for the diurnal variation in adenosine clearance, the ADK inhibitor Iodotubericidin was applied prior to superfusion of adenosine. Blocking this enzyme eliminated the wakefulness-dependent difference in

adenosine clearance (Fig. 3.5C) demonstrating that the observed diurnal variation requires ADK activity.

Taken together, these results suggest that astrocytes provide a major source of extracellular adenosine which increases in response to wakefulness. This adenosine modulates synaptic transmission in multiple brain regions, including the hippocampus and cortex suggesting that it may be responsible for changes in network activity and function in these areas associated with increased sleep pressure following wakefulness. This wakefulness-dependent increase requires SNARE dependent astrocytic release of adenosine and is blocked by inhibiting exocytosis from these cells. Interestingly, there appears to be an opposing increase in the rate of clearance which occurs at the end of the subjective day through an increase in the rate of adenosine kinase activity. This observation suggests that adenosine regulation may be fine-tuned by multiple processes to ensure consistent modulation by this gliotransmitter.

Chapter 4: Chronic sleep restriction disrupts sleep homeostasis and behavioral sensitivity to alcohol

Contributing Authors: Clasadonte, J. and McIver, S. performed experiments included in this chapter. Halassa M. was consulted during the design of this research.

Section 4.1: Overview

Sleep impairments are comorbid with a variety of neurological and psychiatric disorders including depression, epilepsy and alcohol abuse. Despite the prevalence of these disorders, the cellular mechanisms underlying the interaction between sleep disruption and behavior remain poorly understood. To better understand this relationship we studied the impact of accumulated sleep loss on homeostatic sleep regulation by examining markers of sleep pressure in C57BL/6J mice following 72 hours of sleep restriction. Specifically the electroencephalographic power of slow-wave activity (SWA; 0.5-4 Hz) in non-rapid eye movement (NREM) sleep was examined and adenosine tone was measured during and after sleep restriction, as well as following subsequent acute sleep deprivation. During the first day of sleep restriction, SWA and adenosine tone increased, consistent with a homeostatic response to sleep loss. On subsequent days SWA declined to baseline while adenosine tone decreased below baseline and remained low for at least two weeks. Furthermore, the response to acute (6 h) sleep deprivation was significantly attenuated in previously sleep restricted mice.

To investigate the behavioral consequences of chronic sleep restriction, sensitivity to the motor-impairing effects of alcohol was also examined. Sleep restricted mice were significantly less sensitive to the motor-impairing effects of alcohol when tested 24 h after sleep restriction, an effect that persisted for up to 4 weeks. Intracerebroventricular infusion of an adenosine A1 receptor antagonist produced a similar decrease in sensitivity

to alcohol. These results suggest that chronic sleep restriction induces a sustained impairment in adenosine-regulated sleep homeostasis and consequentially impacts the response to alcohol, which is known to involve adenosine signaling.

Section 4.2: Specific Methods

Electroencephalogram/electromyogram implantation surgery: Mice were anaesthetized 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 follows: two extradural cortical electrodes were inserted bilaterally in the frontal areas and the other two were inserted bilaterally in the parietal areas. For implantation of electromyogram (EMG), two insulated wire electrodes were inserted bilaterally into the nuchal muscle. Electrodes connected to a microconnector were secured at the surface of the skull with dental acrylic.

After surgery, mice were intraperitoneally injected with buprenorphine (0.08 mg/kg) and lactated Ringer's solution, and fed moistened rodent food. After 5 days of post-operative recovery, lightweight recording cables were connected to the head implants and mice were placed on a treadmill in individual bottomless Plexiglass cages containing water and food ad libitum and acclimated for a week, as described above. EEG and EMG signals were band-pass filtered at 0.3-100 Hz and 10-100 Hz, respectively, using a 15 LT Bipolar Physiodata amplifier system (Grass Technologies), sampled at 200 Hz with a MP150 data acquisition system (BIOPAC Systems) and stored on a personal computer. Following acclimation, EEG/EMG activity was continuously monitored for 6 days. Baseline EEG/EMG activity starting at ZT0 was measured for 24 h

(baseline), followed by 72 h of sleep restriction (3 days: SR1, SR2 and SR3). Following sleep restriction, recovery EEG/EMG activity was monitored for 24 h. For the acute sleep deprivation post-sleep restriction procedure (see Fig. 4.3A), the treadmill was engaged for 6 more hours (from ZT0 to ZT6) beginning 24 h after termination of sleep restriction. EEG/EMG activity was monitored during the 6 h of sleep deprivation and 18 h of recovery sleep. Sleep deprived mice housed on the treadmill but not subjected to 3 days of sleep restriction (non-sleep restricted group) were used as controls.

Vigilance state scoring and analysis: Sleep stages were scored visually based on 4 s epochs by a trained experimenter using SleepSign for Animal software (Kissei Comtec). Wakefulness (W) 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-rapid eye movement (NREM) sleep consisted of high-amplitude, low frequency EEG with little EMG modulation. Brief awakenings defined as uninterrupted waking episodes of 1-4 s 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 1 hour time bins) and their duration were measured. During the 6 h acute sleep deprivation procedure, NREM sleep latency was defined as the time elapsed between the beginning of the 18 h of recovery sleep and the first NREM sleep episode. EEG power spectra of consecutive 4 s epochs (fast Fourier transform routine; Hanning window) were calculated. The EEG power during NREM sleep from 0.5-4.0 Hz was defined as slow-wave activity (SWA)

and was used as a quantitative measure of sleep pressure and homeostatic sleep drive (Steriade, 2005). The EEG power of SWA during NREM sleep recorded for the 6 consecutive days was expressed as a percentage of the individual mean SWA power in NREM sleep over the entire 24 h of the baseline period, except where otherwise noted. This transformation was conducted to correct for individual differences in the absolute power.

Extracellular recordings: Following recovery incubation, a hippocampal slice was transferred to a recording chamber continuously superfused at 1.2 ml/min with artificial cerebrospinal fluid (aCSF) containing the following: 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM $NaHPO_4$, 10 mM Glucose,1 mM Sodium Pyruvate, 2.9 mM KCl, 2mM $CaCl_2$ and 1 mM $MgCl_2$). The CA1-CA2 boundary was severed to minimize spontaneous recurrent activity, and a glass electrode containing aCSF was positioned in area CA1 for field excitatory postsynaptic potential (fEPSP) recordings. The signal was amplified and band pass filtered between 0.1 Hz and 1 kHz using a 1800 microelectrode amplifier (A-M Systems, Sequim, WA) and digitized at 5 kHz using a Digidata 1320 digitzer (Molecular Devices). All recordings were made at 32.8°C and bath temperature was maintained using an inline heater and bath heating elements (Harvard Instruments).

Throughout recordings paired stimulation pulses were delivered using a 125 μm concentric Platinum-Iridium electrode in the stratum radiatum to orthodromically stimulate axons entering area CA1. Pulses were separated by a 25 ms interstimulus interval unless otherwise noted. Stimulation and recording sweeps were made at 0.033

Hz frequency. Recordings were stored using Clampex 9.2 software and analyzed using Clampfit (Molecular Devices).

Tonic adenosine A1 receptor mediated inhibition (adenosine tone) and adenosine reuptake rate measurement: The slope of the fEPSP was measured at the CA3-CA1 Schaeffer collateral synapse, and stimulus intensity/response curves were plotted for each slice using stimulating currents of increasing intensity ranging from 50 and 175 μA. The maximum slope obtained was used to estimate the point of saturation, and the stimulus intensity required to produce half the maximum slope was estimated and used as the stimulation intensity for the baseline and subsequent recordings. Input/output scatterplots were made by plotting the fEPSP slope at increasing stimulation intensities against the peak amplitude of the fiber volley. Input/Output curve slopes for each slice were determined using a linear regression fit. Paired pulse recordings were made throughout the experiment with an interstimulus interval of 25 ms within each sweep. After a 30 min baseline period at half maximal stimulation, the adenosine type 1 receptor- (adoA1R) mediated inhibitory tone was measured by perfusing the slice with the A1R antagonist 8 -cyclopentyl-1,3-dimethylxanthine (CPT, 200 nM, Sigma) and measuring the alleviation of inhibition according to the following equation: Adenosine Tone = ([fEPSP.cpt - fEPSP.acsf] / fEPSP.acsf). This concentration was chosen because it is consistent with previous studies (Halassa et al. 2009, Arrigoni et al., 2001; Schmitt et al., 2012) and is considered to be selective in this range for A1R (Ki = 10.9 nM) over A2R (Ki = 1440 nM) (Bruns, 1981). Sensitivity to A1R activation was estimated *in situ* using the A1R specific agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA, Sigma) applied to

increasing concentrations at 15 min intervals. Passive reuptake of adenosine was measured indirectly by blocking the equilibrative nucleoside transporter 1 (ENT1) using the transport inhibitor 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR, 100 nM). This concentration was chosen based on previous studies showing that it was sufficient to inhibit synaptic transmission through elevation of adenosine (Pascual et al. 2005). Following a 15 min baseline, NBMPR was applied for 20 min and then washed out for at least 20 min. To determine whether the effect of NBMPR depended on adenosine A1 receptors, these experiments were repeated in the presence of CPT (200 nM). In these experiments, CPT was applied for 30 min prior to the baseline recording after which the experiment was conducted using the same procedure as described above.

Assessment of alcohol sensitivity: Acute sensitivity to the motor-impairing effects of alcohol was measured following intraperitoneal (i.p.) administration of ethanol (2.0 g/kg; 20 ml/kg volume). Mice were first trained to walk on a rotarod apparatus at a speed of 22 rpm until they were able to reach an arbitrary baseline of 60 s (latency to fall >60 s). The following day, mice were tested again to confirm a baseline performance of 60 s, then injected with ethanol and returned to their home cages. The latency to fall was measured 15, 30, 45, 60 and 80 min post-injection, and performance was plotted based on percentage of baseline performance. There was no difference in baseline performance between sleep restricted and non-sleep restricted mice.

Measurement of blood alcohol content: At the end of the rotarod tests (approximately 90 min after the alcohol injection), blood samples were obtained using submandibular

puncture and quickly placed on ice. Blood serum was isolated and further analyzed for blood alcohol content using the Ethanol Assay (Sekisui/Genzyme Diagnostics; Framingham, MA). Analysis was carried out according to the manufacturer's protocol.

In vivo pharmacology: Alzet micro-osmotic pumps (Model 1002; flow rate 0.24µl/hour; DURECT Corporation; Cupertino, CA) were filled with 100 µM 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; Sigma; St. Louis, MO) or vehicle (aCSF + 0.1% DMSO), sealed off with a closed flow moderator and incubated overnight in 0.9% saline. Immediately prior to surgery the pump and flow moderator were connected to catheter and brain infusion cannula, each primed with DPCPX or vehicle, according to the manufacturer's instructions (DURECT Corporation; Cupertino, CA). One pump per animal was subcutaneously implanted along the left dorsal side of the anesthetized mouse and the cannula was stereotaxically targeted to the left cerebral ventricle (A/P, −0.6 mm; M/L, −0.5 mm), and secured with dental cement. The scalp was sutured over the cannula and mice were monitored daily thereafter. Mice were trained and tested on the rotarod 10-12 days after surgery.

Alcohol Recovery Rate Analysis: To measure the 50% recovery rate, data from 15 min – 80 min post-alcohol injection was fit to a sigmoidal curve and the V50 was calculated using GraphPad Prism 5. For all comparisons, the level of significance was set at $p < 0.05$. Data are presented as mean ± SEM.

Section 4.3: Results

Intermittent treadmill activity effectively restricts sleep and disrupts sleep architecture

After one week acclimation to the treadmill, hypnograms obtained from EEG/EMG monitoring on the baseline day indicated normal sleep architecture, with mice primarily sleeping during the light phase (Fig. 4.1 A-C). Throughout the 3 days of intermittent treadmill activity, however, sleep was almost exclusively restricted to each 2 h period when the treadmill was not active (OFF; Fig. 4.1 A-C), with amount of time spent in NREM, REM and total sleep (NREM + REM sleep) reduced by 58.03%, 47.74% and 56.87%, respectively (Fig. 4.1 D-F). Compared to baseline, there was a moderate but significant increase in total sleep time, accounted for solely by NREM sleep, during the recovery day following sleep restriction (Fig. 4.1 D-F). Collectively, these results demonstrate that intermittent treadmill activity alters overall sleep architecture and serves as an effective method of sleep restriction.

Chronic sleep restriction impairs the wakefulness-induced homeostatic increase in slow-wave activity

The spectral power of the EEG in the frequency range of 0.5-4 Hz during NREM sleep (slow-wave activity, SWA) provides a direct metric for sleep need—it increases during normal wakefulness and with extended wakefulness (sleep deprivation; Steriade, 2005). Slow oscillations are thought to be driven by local neocortical circuitry, and therefore, are dependent on intracortical synaptic strength (Fellin et al, 2009). Given that homeostatic

Figure 4.1:

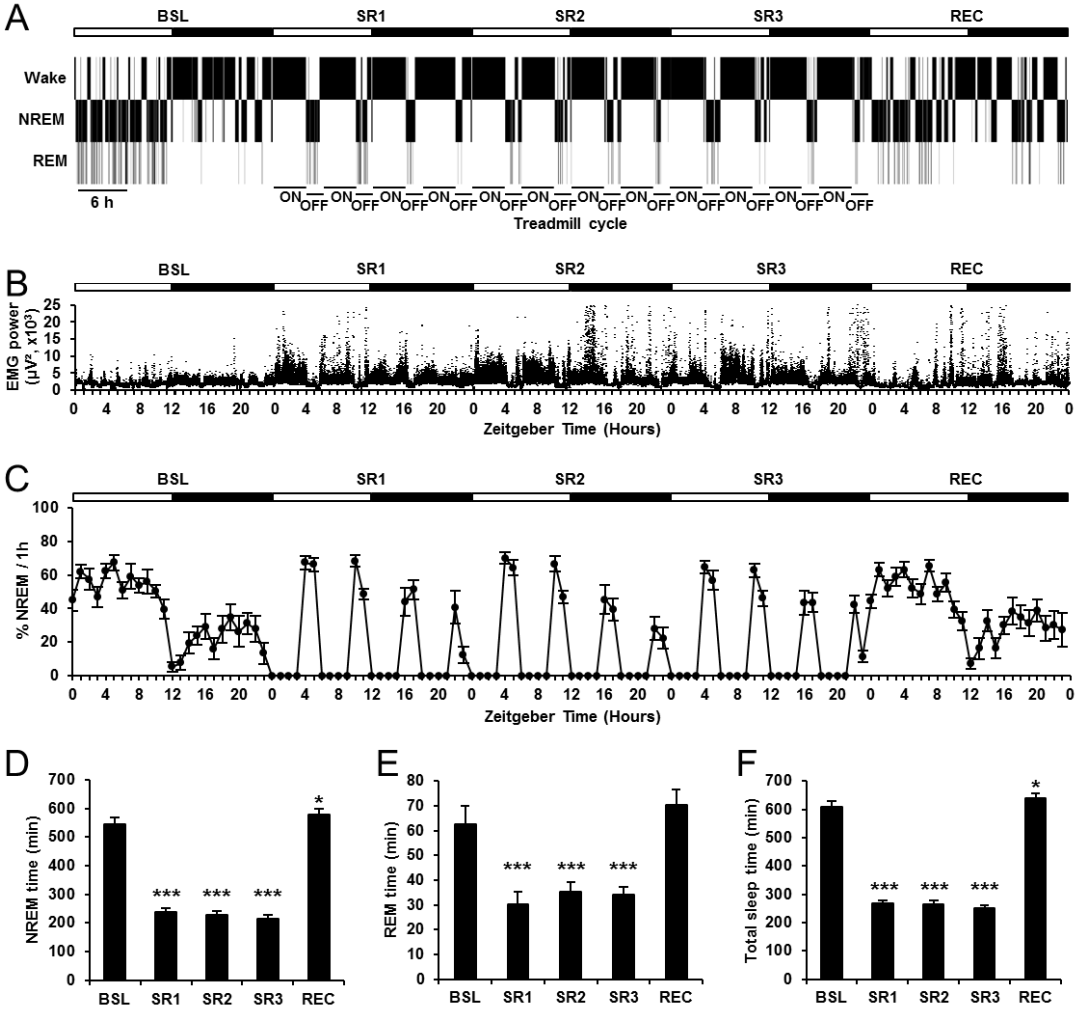

Figure 4.1: Chronic sleep restriction affects sleep architecture. **(A)** Representative hypnogram of a mouse housed on a treadmill during baseline day (BSL), 3 consecutive days of sleep restriction (SR1, SR2 and SR3) and recovery day (REC). The slow-moving treadmill was on for 4 h (ON) and off for 2 h (OFF) for a total of 12 complete 6 h cycles. Mice were maintained on a 12 h light (open 12 h horizontal bar)/12 h dark (filled 12 h horizontal bar). Note the disruption of the circadian pattern of the NREM sleep during the 3 consecutive days of sleep restriction compared with the baseline and recovery days. **(B)** Analysis of EMG power from the mouse presented in (A). Note the increase in EMG power during the corresponding periods of spontaneous and enforced wakefulness. **(C)** Percentage average of the time spent in NREM sleep per hour during the 5 days of the experiment on the treadmill (n = 11 animals). **(D-E)** Averaged amount of time spent in NREM sleep (D) and REM sleep (E) during each 24 h of the experiment (graph D: one-way repeated measures ANOVA, $F = 295.81$, $p < 0.001$, SNK post-hoc test, $^{*}p < 0.05$, $^{***}p < 0.001$ when compared to BSL, n = 11 animals; graph E: one-way repeated measures ANOVA, $F = 30.08$, $p < 0.001$, SNK post-hoc test, $^{***}p < 0.001$ when

compared to BSL, n = 11 animals). **(F)** The averaged amount of total sleep time (NREM + REM) during each 24 h of the experiment was significantly decreased during the 3 day period of chronic sleep restriction (one-way repeated measures ANOVA: $F = 350.06$, $p < 0.001$; SNK post-hoc test, $^{*}p < 0.05$, $^{***}p < 0.001$ when compared to BSL; n = 11 animals). This work was performed by Jerome Clasadonte.

sleep drive is intimately related to wake-dependent synaptic potentiation (Hanlon et al., 2009), the cortical slow oscillation is considered a read-out for sleep homeostasis (Vyazovsiky et al., 2008; Vyazovsiky et al., 2009). During the first day of sleep restriction, there was a significant increase in the power of SWA in NREM sleep recorded during TM-off periods, consistent with accumulated sleep pressure during enforced wakefulness; this increase was significantly attenuated during the subsequent days of sleep restriction and returned to baseline on the recovery day (Fig. 4.2 A-C). Though SWA power was unaltered in REM sleep (data not shown), it was moderately increased during wake (Fig. 4.2 D-G), especially when the treadmill was active (Fig. 4.2 F, G; TM-on), suggesting that sleep restriction induced breakthrough sleep, or SWA leakage during periods of enforced wakefulness, consistent with previous studies (Leemburg et al., 2010). There was also a significant increase in theta power (6-10 Hz) during wake throughout the course of sleep restriction (Fig. 4.2 F; TM-on). Because wake was enforced using the treadmill, this is likely a result of continuous motor activity, which is known to be associated with theta power (Vanderwolf, 1969; Whishaw and Vanderwolf, 1973).

Because the progressive attenuation in the increases of NREM SWA during the 2-h sleep opportunites (TM-off) could result from a compensatory increase in SWA accumulated across the 4-h enforced wakefulness procedure (TM-on), we computed the cumulative integrated SWA power throughout the 24 h of each day of the experiment

(summed SWA power over all epochs during TM-off and TM-on periods) in NREM, wake and all vigilance states combined (Fig. 4.3 A-C). A cumulative loss of integrated SWA was observed during the 3 days of sleep restriction in NREM (Fig. 4.3 B) when compared to the baseline day whereas there was no change in wake (Fig. 4.3 A). In addition, although the cumulative integrated SWA in wake was similar across the 3 days of sleep restriction (Fig. 4.3 A), it was significantly attenuated in NREM on the third day of sleep restriction when compared to the first day (Fig. 4.3 B). The progressive loss of cumulative integrated SWA during the 3 days of sleep restriction was conserved when values for integrated SWA power during both wake and REM were added to that for NREM (Fig. 4.3 C). These data suggest that the attenuation in NREM SWA during the TM-off periods (Fig. 4.2 A-C) was not a consequence of a compensatory increase in cumulative SWA during the course of sleep restriction periods (TM-on). In addition, we observed that the cumulative integrated SWA during the recovery day in wake, NREM and all vigilance states was significantly higher than during the baseline day (Fig. 4.3 A-C), suggesting that the homeostatic sleep pressure cumulated during the 3 days of sleep restriction was dissipating after sleep restriction.

Furthermore, we computed the cumulative integrated SWA power throughout the entire experiment (before, during and after chronic sleep restriction). Consistent with previous studies (Deurveilher et al., 2012), the cumulative integrated SWA in NREM and all vigilance states during recovery paralleled that during baseline (Fig. 4.3 D-F), suggesting that the dissipation of homeostatic sleep pressure seen during recovery (Fig. 4.3 A-C) was not sufficient to recover the cumulative loss of integrated SWA after sleep restriction. Collectively these findings show that chronic sleep restriction alters sleep

Figure 4.2:

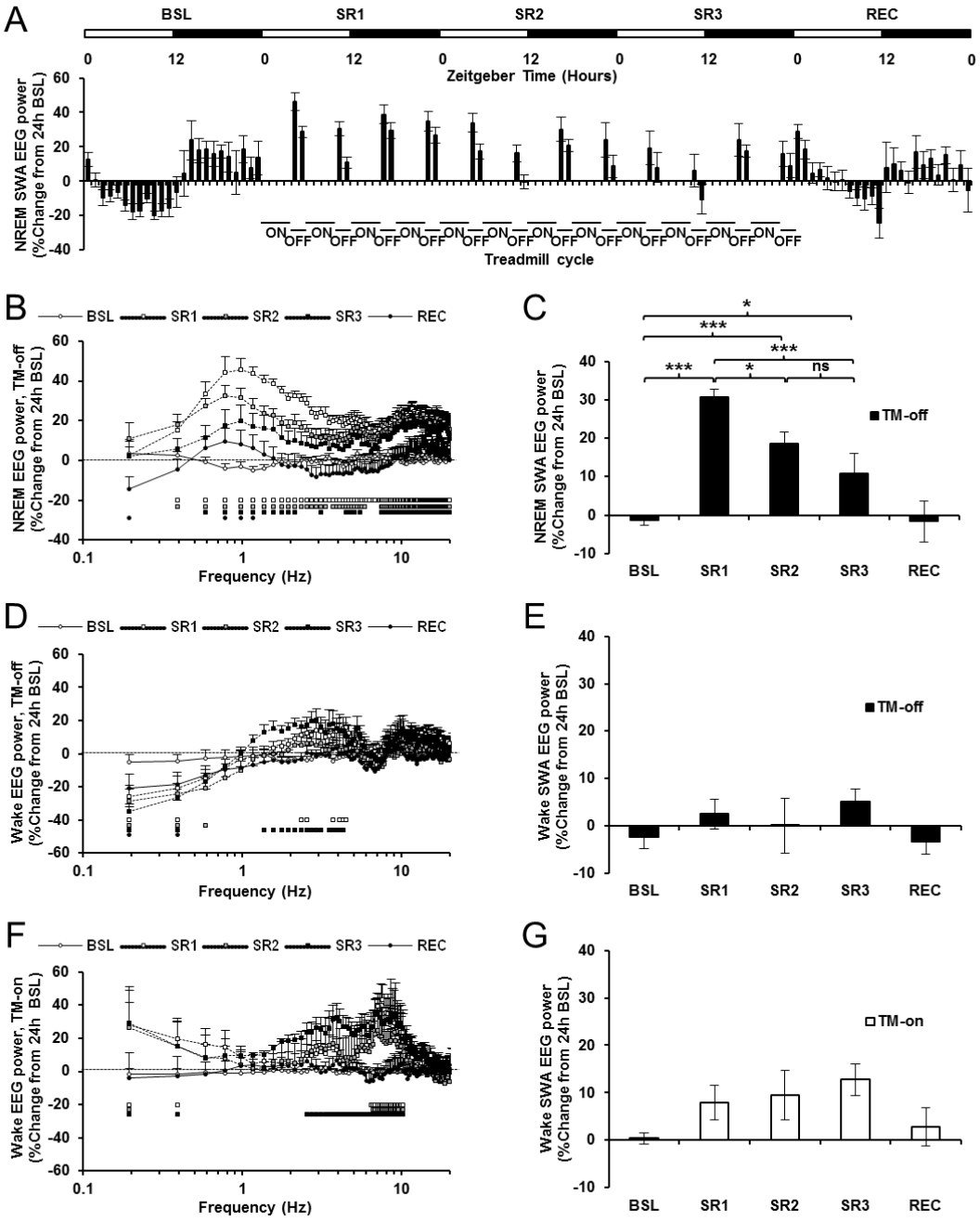

Figure 4.2: Effect of chronic sleep restriction on slow-wave activity. **(A)** EEG power of slow-wave activity (SWA: 0.5-4 Hz) during NREM sleep for each hour of the 5 days of the experiment on the treadmill plotted as percentage of 24 h mean values of the baseline day (n = 11 animals). Note the increase in SWA power during the 2 h TM-off periods. **(B)** NREM sleep EEG power during the total 8 h TM-off periods for each day of the experiment on the treadmill plotted as percentage of 24 h mean values of the baseline day

(two-way repeated measures ANOVA: day, $F = 6.84$, $p < 0.001$; frequency, $F = 6.28$, $p < 0.001$; day x frequency, $F = 4.60$, $p < 0.001$; $n = 11$ animals). Squares and circles at the bottom indicate frequencies that significantly differ from the baseline (SNK post-hoc test, $p < 0.05$). **(C)** EEG power of SWA (% change from 24 h baseline) in NREM sleep during the total 8 h TM-off periods for each day of the experiment on the treadmill. SWA during the baseline and recovery days were averaged over the corresponding total 8 h TM-off periods of each day of sleep restriction. Note the progressive attenuation in the increase of SWA over the 3 days of sleep restriction (one-way repeated measures ANOVA: $F = 17.69$, $p < 0.001$; SNK test, *$p < 0.05$, ***$p < 0.001$; $n = 11$ animals; ns, nonsignificant). **(D)** Wake EEG power during the total 8 h TM-off periods for each day of the experiment on the treadmill plotted as percentage of 24 h mean values of the baseline day (two-way repeated measures ANOVA: day, $F = 0.93$, $p > 0.05$; frequency, $F = 2.42$, $p < 0.001$; day x frequency, $F = 2.47$, $p < 0.001$; $n = 6$ animals). Squares and circles at the bottom indicate frequencies that significantly differ from the baseline (SNK post-hoc test, $p < 0.05$). **(E)** EEG power of SWA (% change from 24 h baseline) in wakefulness during the total 8 h TM-off periods for each day of the experiment on the treadmill. SWA during the baseline and recovery days were averaged over the corresponding total 8 h TM-off periods of each day of sleep restriction (one-way repeated measures ANOVA, $F = 0.96$, $p > 0.05$, $n = 6$ animals). **(F)** Wake EEG power during the total 16 h TM-on periods for each day of the experiment on the treadmill plotted as percentage of 24 h mean values of the baseline day (two-way repeated measures ANOVA: day, $F = 0.97$, $p > 0.05$; frequency, $F = 2.91$, $p < 0.001$; day x frequency, $F = 3.34$, $p < 0.001$; $n = 6$ animals). Squares at the bottom indicate frequencies that significantly differ from the baseline (SNK post-hoc test, $p < 0.05$). **(G)** EEG power of SWA (% change from 24 h baseline) in wakefulness during the total 16 h TM-on periods for each day of the experiment on the treadmill. SWA during the baseline and recovery days were averaged over the corresponding total 16 h TM-on periods of each day of sleep restriction (one-way repeated measures ANOVA, $F = 2.87$, $p > 0.05$, $n = 6$ animals). This work was performed by Jerome Clasadonte.

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physiology and leads to an impairment of sleep homeostasis, marked by a dampening of sleep pressure or SWA accumulation.

To test whether the sleep homeostat could be re-activated following chronic sleep restriction, mice were subjected to acute (6 h) sleep deprivation following one day of recovery from sleep restriction (Fig. 4.4 A). Six hours of treadmill activity effectively prolonged wakefulness in both previously undisturbed and sleep restricted mice (data not shown), and induced an increase in SWA during recovery sleep, which was significantly

Figure 4.3:

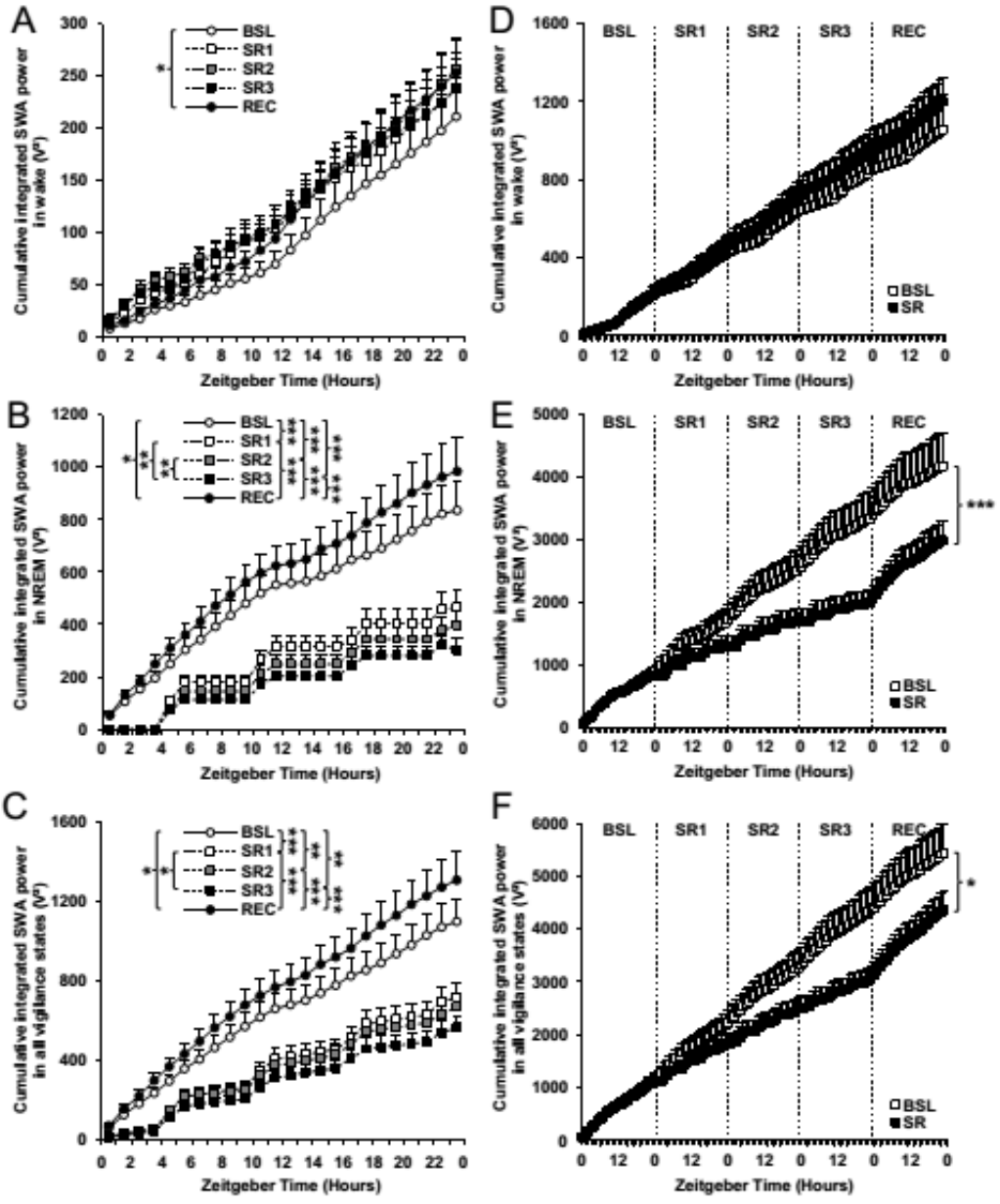

Figure 4.3: Cumulative loss of integrated slow-wave activity does not fully recover after chronic sleep restriction. **(A-C)** Time course of integrated slow-wave activity (SWA) cumulated over 24 h is shown in wake (A), NREM (B) and all vigilance states (C). EEG power of SWA was summed in 1-h bins and cumulated over 24 h during the baseline day (BSL), 3 consecutive days of sleep restriction (SR1, SR2 and SR3) and recovery day (REC). Asterisks indicate cumulative curves that significantly differ between days (two-way repeated measures ANOVA followed by SNK post-hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 11 animals). **(D-F)** Time course of integrated SWA cumulated over the

entire experiment in wake (D), NREM (E) and all vigilance states (F). EEG power of SWA was summed in 1-h bins and cumulated over the 5 days of the chronic sleep restriction experiment (SR curve). The accumulation of integrated SWA over all days in the absence of chronic sleep restriction (BSL curve) represents an extrapolation of the empirical curves obtained on the baseline day. Note that the cumulative loss of integrated SWA during NREM (E) and all vigilance states (F) did not recover at the end of the recovery day (REC; two-way repeated measures ANOVA followed by SNK post-hoc test: *$p < 0.05$, ***$p < 0.001$; $n = 11$ animals). This work was performed by Jerome Clasadonte.

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attenuated in sleep restricted mice (Fig. 4.4 B). The latency to NREM sleep during the 18 h recovery after sleep deprivation was increased following chronic sleep restriction, though this effect was not significant (Fig. 4.4 C). However, the increase in amount of NREM sleep time and NREM episode duration that characteristically occur during sleep rebound after prolonged wakefulness were significantly blunted in sleep restricted mice (Fig. 4.4 D, E), suggesting an absence of sleep compensation in these mice. In addition, we observed that the effects of sleep restriction on sleep homeostasis were still present 2 weeks after the termination of sleep restriction (Fig. 4.4 F-I). Collectively, the findings that sleep restricted mice exhibit reduced SWA rebound and lack of sleep compensation in response to acute sleep deprivation suggest that the homeostatic response to prolonged wakefulness is impaired after chronic sleep restriction.

Chronic sleep restriction impairs the homeostatic adenosine response to prolonged wakefulness

Presynaptic inhibition mediated by tonic adenosine acting on A1Rs (adenosine tone) provides basal inhibition of synaptic transmission. The concentration of extrazcellular adenosine and its relative increase in response to wakefulness is correlated with SWA

Figure 4.4:

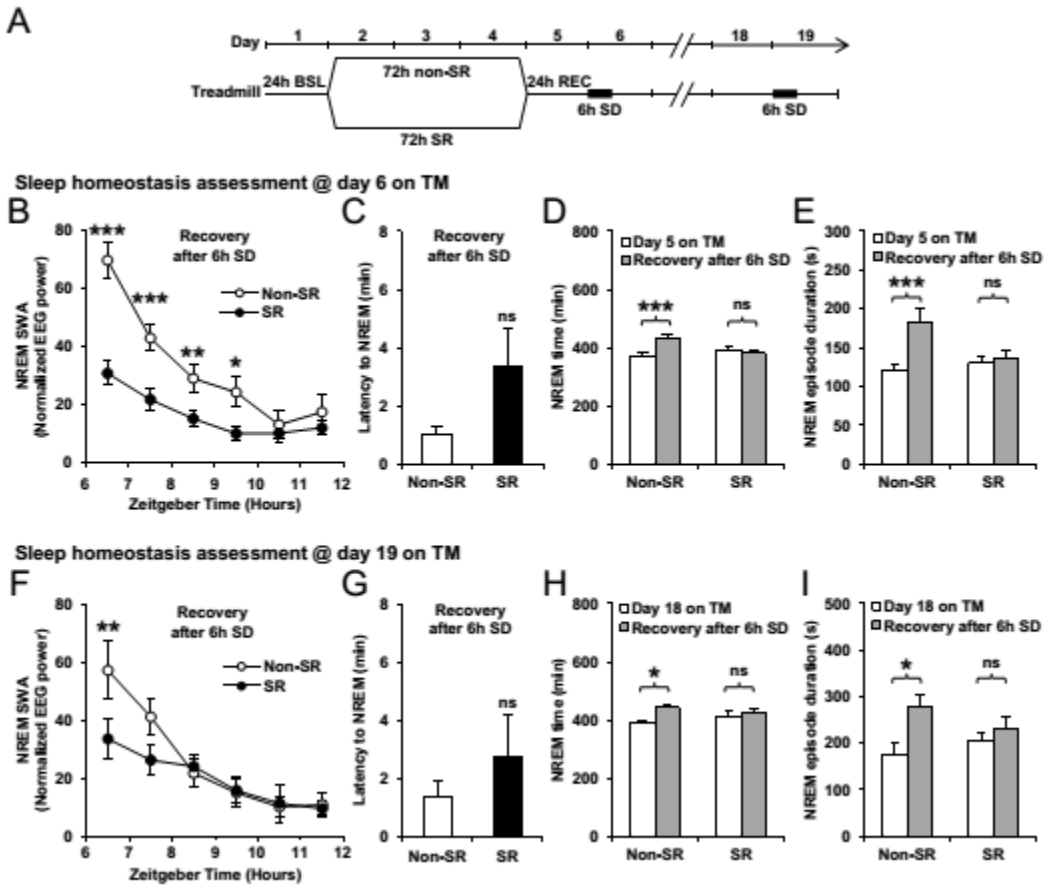

Figure 4.4: Effect of chronic sleep restriction on sleep homeostasis. **(A)** Sleep deprivation (SD) post-sleep restriction schematic: mice were housed on a treadmill and continuously monitored with cortical EEG throughout the entire experiment. Mice were either left undisturbed on the treadmill (non-SR group) or subjected to 3 days (72 h) of sleep restriction (SR group). Assessement of sleep homeostasis was successively conducted on day 6 and day 19 of the experiment for the non-SR group and after 24 h and 2 weeks of recovery for the SR group. Both non-SR and SR mice were sleep deprived for 6 h on the treadmill starting at ZT0 and then left recovering for the following 18 h. **(B-E)** Assessment of sleep homeostasis on day 6. **(B)** Following SD, the increase in SWA power during NREM sleep, expressed as percentage of last 4 h of the light phase during day 5 on the treadmill, was significantly smaller in the SR animals compared to the non-SR animals (two-way repeated measures ANOVA: condition, $F = 13.509$, $p < 0.001$; time, $F = 41.45$, $p < 0.001$; condition x time, $F = 8.02$, $p < 0.001$; SNK post-hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n = 16 animals per condition). **(C)** Average latency to NREM sleep after SD in non-SR and SR animals (Mann-Whitney U test: $U = 98.50$, $p > 0.05$, n = 16 animals per condition; ns, nonsignificant). **(D-E)** SD increased both the amount of time spent in NREM sleep (D) and duration of NREM episodes (E) in non-SR group but not in SR group during the 18 h recovery period compared with the

corresponding 18 h of day 5 on the treadmill (graph D: non-SR group, paired Student's t-test, $t = -4.58$, $^{***}p < 0.001$, SR group, paired Student's t-test, $t = 0.80$, $p > 0.05$, $n = 16$ mice per group; graph E: non-SR group, paired Student's t-test, $t = -4.29$, $^{***}p < 0.001$, SR group, paired Student's t-test, $t = -0.56$, $p > 0.05$, $n = 16$ animals per group; ns, nonsignificant). **(F-I)** Assessment of sleep homeostasis on day 19. **(F)** Following SD, the increase in SWA power during NREM sleep, expressed as percentage of last 4 h of the light phase during day 18 on the treadmill, was significantly smaller in the SR animals compared to the non-SR animals (two-way repeated measures ANOVA: condition, $F = 1.34$, $p > 0.05$; time, $F = 16.78$, $p < 0.001$; condition x time, $F = 2.46$, $p < 0.05$; SNK post-hoc test, $^{**}p < 0.01$; $n = 6$ animals per condition). **(G)** Average latency to NREM sleep after SD in non-SR and SR animals (Unpaired Student's t-test: $t = -0.91$, $p > 0.05$, $n = 6$ animals per condition; ns, nonsignificant). **(H-I)** SD increased both the amount of time spent in NREM sleep (H) and duration of NREM episodes (I) in non-SR group but not in SR group during the 18 h recovery period compared with the corresponding 18 h of day 18 on the treadmill (graph H: non-SR group, paired Student's t-test, $t = -2.86$, $^{*}p < 0.05$, SR group, paired Student's t-test, $t = -1.55$, $p > 0.05$, $n = 6$ mice per group; graph I: non-SR group, paired Student's t-test, $t = -3.33$, $^{*}p < 0.05$, SR group, paired Student's t-test, $t = -1.60$, $p > 0.05$, $n = 6$ animals per group; ns, nonsignificant). This work was performed by Jerome Clasadonte.

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and similarly drives sleep need (Basheer et al., 2004). This wakefulness-dependent increase in adenosine peaks prior to sleep onset and is thought to inhibit cholinergic projections of the brainstem and BF regions to promote sleep. Although similar increases in adenosine have been reported to occur in BF, cortex (Kalinchuk et al., 2011), and more recently, in the hippocampus (Schmitt et al., 2012) in response to spontaneous wakefulness and short term sleep deprivation, the effect of prolonged sleep disruption on adenosine tone is not known.

To examine the impact of chronic sleep restriction on wakefulness-induced accumulation of adenosine, acute hippocampal slices were isolated from mice at multiple time points across the 3 days of sleep restriction. During the first day of sleep restriction (SR1), there was a significant increase in adenosine tone at ZT4, after the first cycle of

Figure 4.5:

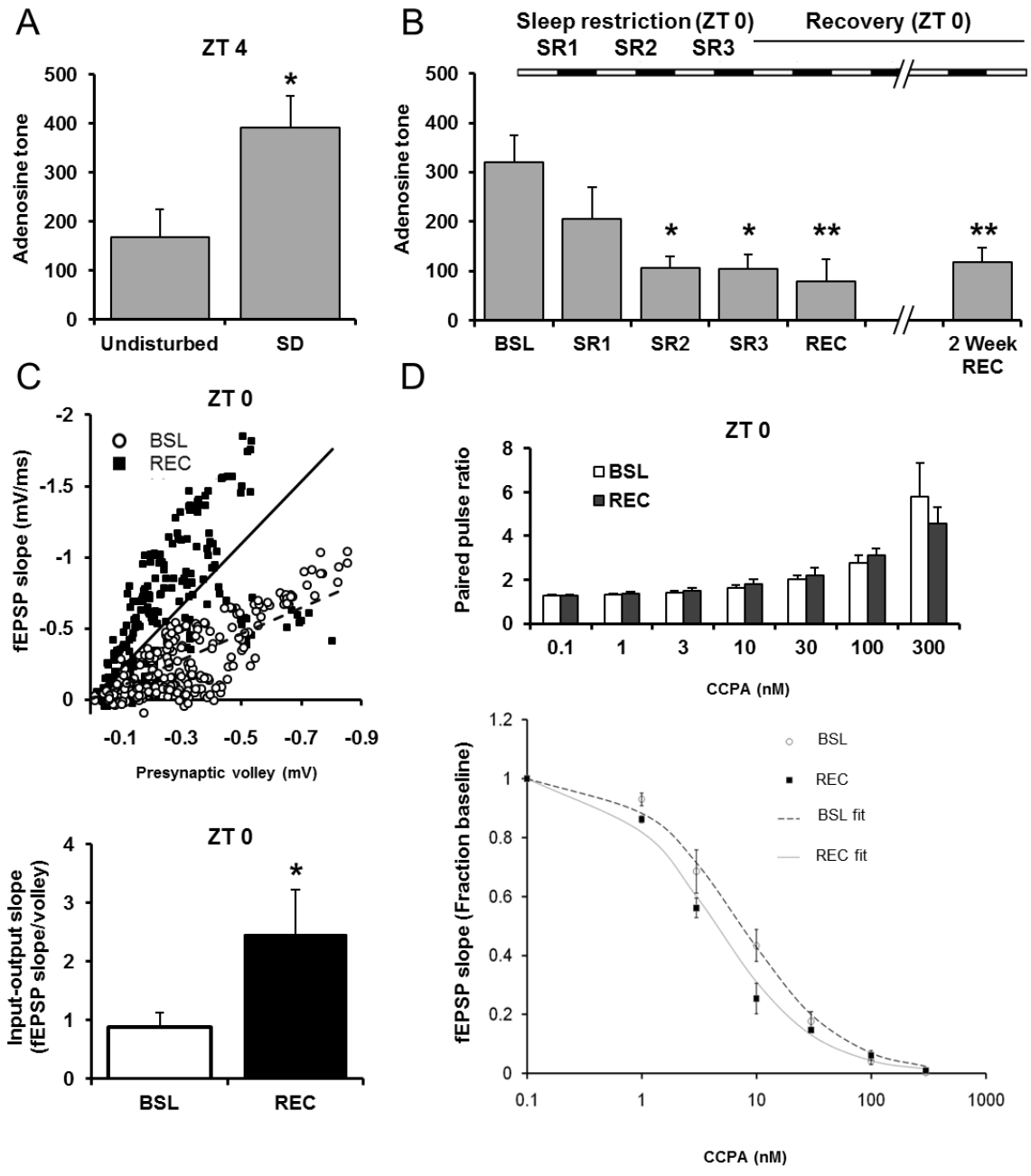

Figure 4.5: Sleep restriction inactivates wakefulness-dependent elevation of extracellular adenosine. **(A)** Adenosine tone was significantly higher in hippocampal slices taken from mice that have been sleep deprived for 4 h on the treadmill compared with slices taken from undisturbed control mice (Mann-Whitney U test: U = 0.00, *p < 0.05; n = 5). **(B)** Compared to baseline undisturbed mice (BSL = 6), 3 consecutive days of sleep restriction (SR1: n = 4; SR2: n = 4; SR3: n = 4) produced a progressive reduction in the level of adenosine tone, measured after each day of sleep restriction at ZT0. This reduced adenosine tone persisted after a recovery period of 24 h (REC, n = 6) and 2 weeks (2

week REC, n = 6) (Group comparison of sleep restriction/recovery with baseline: $p < 0.01$, Kruskal-Wallis ANOVA, H = 10.515; Mann-Whitney post-hoc test, * $p < 0.05$, ** $p < 0.01$). **(C)** Upper panel: The synaptic input/output response measured at ZT0 was reduced in slices from undisturbed mice (BSL) compared with those from sleep restricted mice (REC) (Upper panel). In response to increasing stimulation, the fEPSP slope relative to presynaptic volley showed a greater response to similar axonal input (Linear Fit across recordings, n = 6). Lower panel: The slopes estimated from individual linear fits of input/output curves were significantly increased in sleep restricted mice (REC) relative to undisturbed non-sleep restricted control mice (BSL) (unpaired Student's t-test, t = -2.488, *$p < 0.05$, n = 6). **(D)** Sensitivity to presynaptic inhibition by exogenous activation of A1R with increasing concentrations of the agonist CCPA (from 0.1 to 300 nM) was not reduced in slices taken from sleep restricted mice (REC) compared with slices taken from non-sleep restricted control mice (BSL). Sleep restricted and control mice show similar levels of paired pulse facilitation (upper panel, two-way repeated measures ANOVA: condition, F = 0.069, p = 0.802; concentration, F = 9.927, $p < 0.001$, n = 5 for each group) and synaptic inhibition (lower panel; Hill plot fit of reduction in fEPSP slope relative to baseline; REC: IC50 = 4 nM; BSL: IC50 = 10 nM; n = 5 for each group) for all concentrations of CCPA. The n value indicates the number of animals (1 slice per animal).

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treadmill activity, relative to adenosine levels at ZT4 in undisturbed mice (Fig. 4.5 A), consistent with previous studies in which adenosine tone was increased in mice which were acutely sleep deprived for 4 h using gentle handling (Schmitt et al., 2012). In contrast, chronic sleep restriction reduced the adenosine tone measured at the normal onset of sleep (ZT0) over the subsequent days of sleep restriction (Fig. 4.5 B). Moreover, the reduction in adenosine tone was maintained after 24 h recovery sleep (Fig. 4.5 B); this effect was sustained for at least 2 weeks after the termination of sleep restriction (Fig. 4.5 B). The attenuated level of synaptic inhibition following sleep restriction was also evident in the input/output curve of the baseline fEPSP response (Fig. 4.5 C). In slices taken at ZT0, the fEPSP response as a function of presynaptic volley was greater in sleep restricted mice compared with undisturbed mice (Fig. 4.5 C). The linear fit estimation of the slope of input/output curves from slices at this time point showed a significant increase in slices from sleep restricted mice relative to undisturbed controls (Fig. 4.5 C).

Because adenosine tone depends both on extracellular adenosine levels and A1R surface expression, we next measured whether the reduction was due to a decrease in A1R sensitivity. Dose-response measurements of the A1R agonist, CCPA (0.1 – 330 nM), indicated a modest, though not significant, increase in A1R sensitivity in hippocampi from sleep restricted mice (IC_{50} = 4 nM; Fig. 5D, bottom panel) relative to undisturbed control mice (IC_{50} = 10 nM; Fig. 4.5 D, bottom panel), with no significant change in paired pulse ratios (Fig. 4.5 D; top panel), suggesting that the reduced adenosine tone measurements were not a consequence of down-regulated A1R expression or function, but instead involve a reduction in extracellular adenosine acting at the synapse. Together, these results indicate that sleep restriction prevents the increase in adenosine usually associated with normal wakefulness.

To further test whether sleep restriction disrupts the adenosine response to wakefulness, adenosine tone was measured following 6 h of sleep deprivation in previously undisturbed and sleep restricted mice. Sleep restricted mice were allowed 24 h of recovery after the 3 days of sleep restriction and then sleep deprived by gentle handling for 6 h. The elevation of adenosine tone following 6 h of sleep deprivation was significantly blunted in mice that were previously sleep restricted, compared to undisturbed controls (Fig. 4.6 A). Analysis of the input/output curve for synaptic transmission showed that the field responses to increasing stimulation intensity were dramatically larger relative to the presynaptic volley in the sleep restricted group (Fig. 4.6 B). This observation indicates that the higher inhibition normally observed following

Figure 4.6:

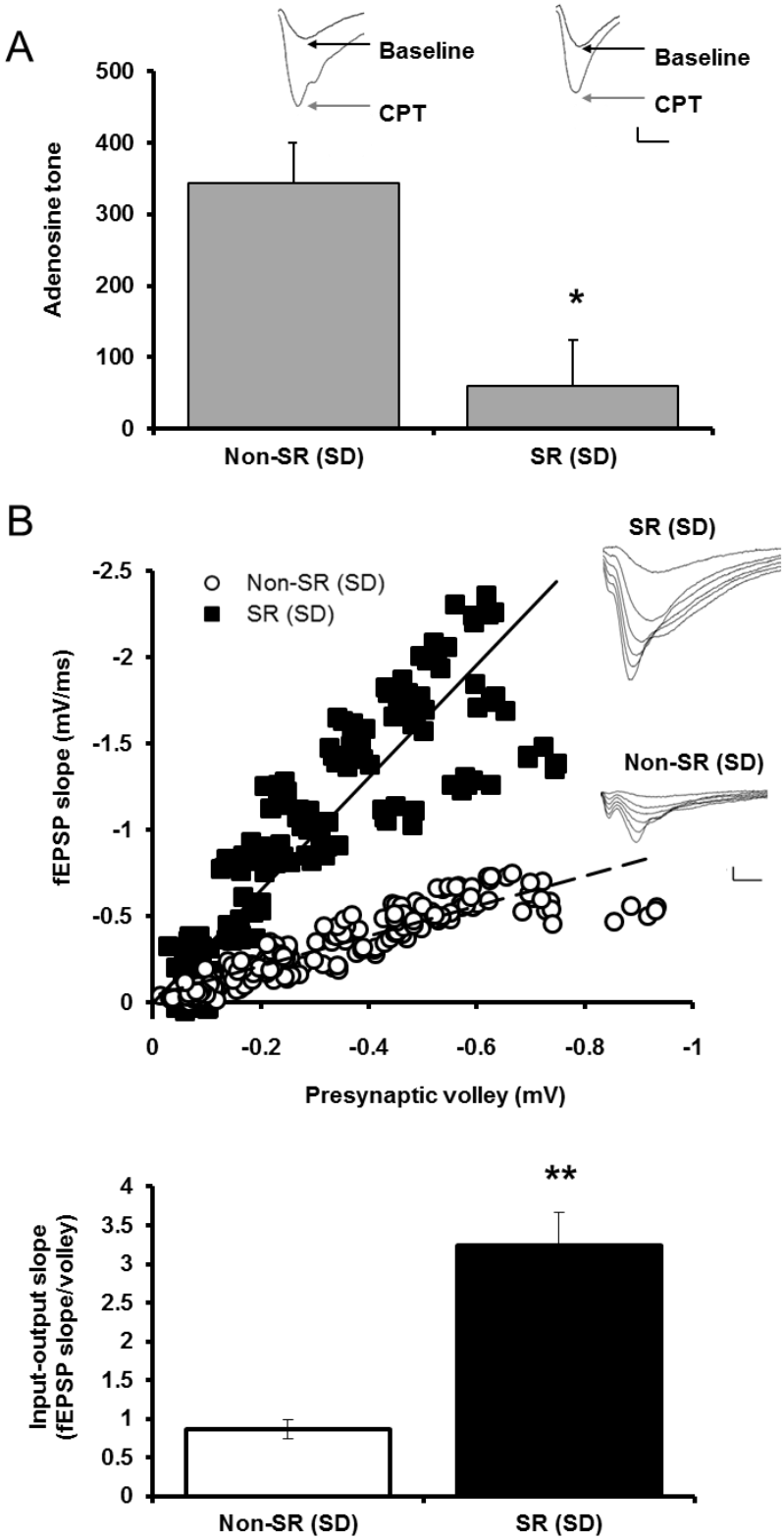
A
Baseline
CPT
Baseline
CPT
Adenosine tone
500
400
300
200
100
0
*
Non-SR (SD)
SR (SD)
B
SR (SD)
Non-SR (SD)
SR (SD)
fEPSP slope (mV/ms)
-2.5
-2
-1.5
-1
-0.5
0
0
-0.2
-0.4
-0.6
-0.8
-1
Presynaptic volley (mV)
Non-SR (SD)
Input-output slope (fEPSP slope/volley)
4
3.5
3
2.5
2
1.5
1
0.5
0
**
Non-SR (SD)
SR (SD)

Figure 6. Prior sleep restriction significantly suppresses the sleep deprivation-dependent increase in adenosine-mediated inhibition. **(A)** Significantly lower adenosine tone is observed in previously sleep restricted (SR) mice compared with non-sleep restricted (Non-SR) controls following 6 hours of sleeps deprivation (Mann-Whitney U test, U = 0.00: **$p < 0.05$, n = 5 per group). Insets represent average of fEPSPs before (baseline) and during application of 200 nM CPT in non-SR (left) and SR mice (right) after 6 h SD. Scale bars: 0.2 mV, 2.5 ms. **(B)** Upper panel: Following sleep deprivation, the input-output curve in previously sleep restricted mice shows an apparent increase compared with controls. In response to increasing stimulation, the fEPSP slope relative to presynaptic volley showed a greater response to similar axonal input in slices from SR mice (Linear Fit across recordings, n = 6 per group). Insets represent average of fEPSPs in response to currents of increasing intensity in non-SR (lower) and SR mice (upper) after 6 h SD. Scale bars: 0.2 mV, 2.5 ms. Lower panel: The slopes estimated from individual linear fits of input/output curves were significantly increased in sleep restricted mice (SR) relative to non-sleep restricted controls (Non-SR) (Tukey's HSD test, q = 3.613 , *$p < 0.05$, n = 6 per group). The n value indicates the number of animals (1 slice per animal).

sleep deprivation (Schmitt et al., 2012) was prevented by prior sleep restriction and further suggests that no increase in other inhibitory pathways occurs to compensate for the loss of wakefulness-dependent adenosine.

The lower level of wakefulness-dependent adenosine produced by sleep restriction could either be caused by a reduction in the sources of adenosine or by increased clearance. Removal of adenosine from the extracellular space is primarily mediated by passive re-uptake through the glial equilibrative nucleoside transporter type 1 (ENT1) (Wall and Dale, 2013). To determine whether changes in the rate of clearance through this pathway were responsible for the lower level of tonic adenosine following sleep restriction, we selectively blocked ENT1 with NBMPR (100 nM) and measured the effect on adenosine mediated-synaptic inhibition.

Consistent with previous results (Pascual et al., 2005), in slices from undisturbed mice we observed an inhibition of synaptic transmission in response to NBMPR (Fig. 4.7 A). This effect was blocked in the presence of CPT (Percentage change in fEPSP slope;

Figure 4.7:

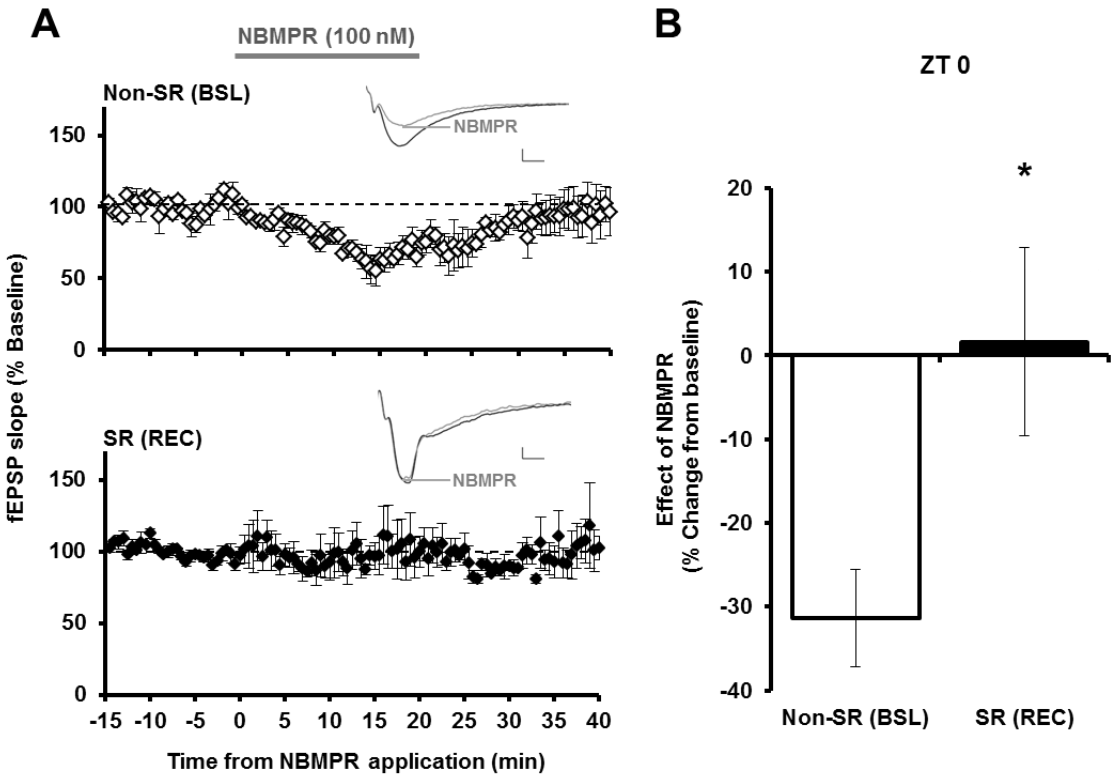

Figure 7. Passive reuptake of adenosine is reduced following sleep restriction. **(A)** Blocking passive transport of adenosine through ENT1 with NBMPR (100 nM) produces a marked reduction in fEPSP slope in control slices from undisturbed mice (Non-SR) which was not evident following sleep restriction (SR). Insets represent average of fEPSPs before (black trace) and during application of 100 nM NBMPR (grey trace) in non-SR (upper graph) and SR mice (lower graph). Scale bars: 0.2 mV, 5 ms. **(B)** Average percentage change of the fEPSP slope in response to NBMPR calculated during the last 5 min of the application period. A significantly greater change in synaptic inhibition was observed in non-SR slices compared to those from the SR group (Tukey's HSD test, $q = 4.271$ *$p < 0.05$, $n = 5$ per group). The n value indicates the number of animals (1 slice per animal).

without CPT: - 31.34 ± 5.79 %, n = 6 slices vs. with CPT: 12.91 ± 6.73 % n = 5 slices; Tukey's HSD test, $q = 3.27$, $p < 0.05$), indicating that the synaptic inhibition was due to A1R activation. If increased clearance by passive reuptake through ENT1 is responsible for the reduction in extracellular adenosine by sleep restriction, blocking this transporter should lead to a higher level of A1R-mediated synaptic inhibition. Surprisingly, we instead observed a significantly smaller synaptic inhibition in slices from sleep restricted mice (Fig. 4.7 A, B), suggesting that the lower adenosine tone following sleep restriction is likely due to a reduction in the source of adenosine. Together, these results support the hypothesis that the allostatic reduction in homeostatic sleep pressure by sleep restriction occurs because wakefulness-dependent changes in the sources of adenosine are dampened.

Sensitivity to alcohol is significantly reduced following chronic sleep restriction

Recent studies have suggested a role for adenosine signaling pathways in the mechanisms underlying the bidirectional relationship between sleep impairments and alcohol abuse (Nam et al., 2012). Because it is known that alcohol increases adenosine levels (Nagy et al., 1990), which in turn, mediate the motor-impairing effects of alcohol via A1R activation (Barwick and Dar, 1998; Dar, 2001), we asked whether the sustained reduction in adenosine tone seen in sleep restricted mice could be correlated with a reduction in the behavioral response to alcohol. Sensitivity to the motor-impairing effects of alcohol was measured 24 h, 2 weeks and 4 weeks after termination of sleep restriction. All mice were able to remain on the rotarod for at least 60 s (baseline) prior to receiving an intoxicating dose of alcohol (2.0 g/kg, i.p.). The latency to fall was measured at 15 min intervals up to

Figure 4.8:

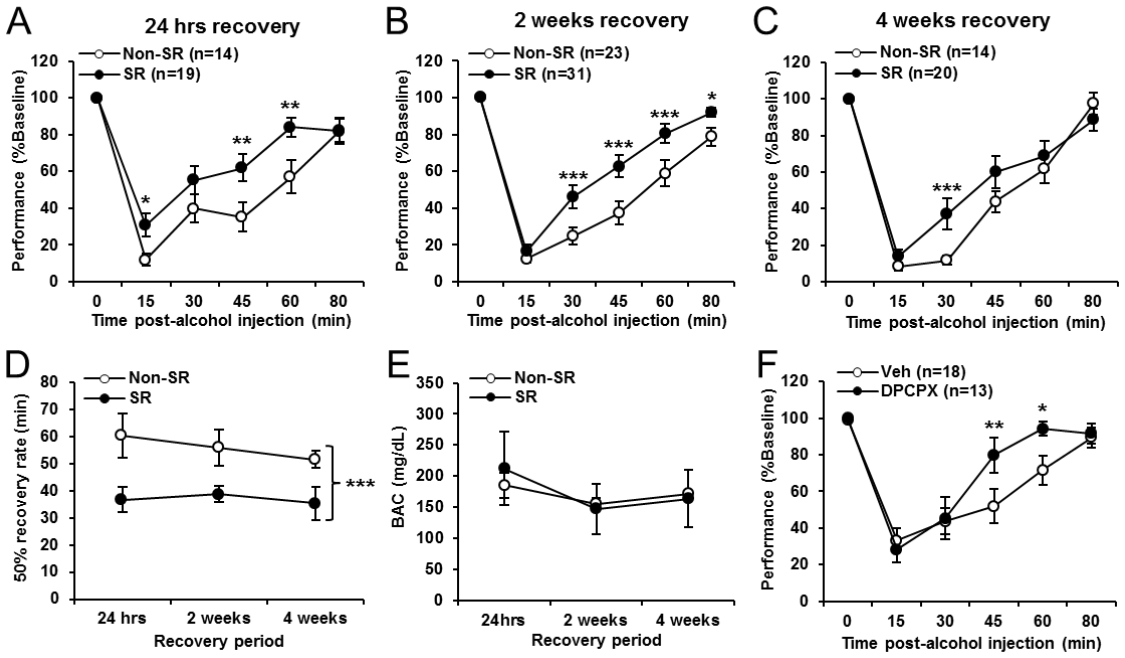

Figure 4.8: Sensitivity to the motor-impairing effects of alcohol is decreased following chronic sleep restriction. **(A)** Sleep restricted mice (SR) were significantly less impaired on the rotarod after alcohol injection (2 g/kg) compared with non-sleep restricted control mice (Non-SR) when tested 24 hrs after termination of sleep restriction (two-way repeated measures ANOVA: condition, $F = 7.005$, $p < 0.05$; time, $F = 51.617$, $p < 0.001$; condition x time, $F = 2.401$, $p < 0.05$; Bonferonni post-hoc test, *$p < 0.05$, **$p < 0.01$). **(B-C)** The effect persisted when mice were tested 2 weeks later (B, two-way repeated measures ANOVA: condition, $F = 13.208$, $p < 0.001$; time, $F = 117.973$, $p < 0.001$; condition x time, $F = 3.119$, $p < 0.01$; Bonferonni post-hoc test, *$p < 0.05$, ***$p < 0.001$) and 4 weeks later (C, two-way repeated measures ANOVA: condition, $F = 4.915$, $p < 0.05$; time, $F = 72.375$, $p < 0.001$; Bonferonni post-hoc test, ***$p < 0.001$). **(D)** Compared to non-SR mice, the time required for mice to reach 50% recovery rate is significantly less in SR mice (two-way repeated measures ANOVA: condition, $F = 15.308$. ***$p < 0.001$). **(E)** Blood alcohol levels measured approximately 90 min after ethanol injection were not significantly different between SR and non-SR control mice at any time point measured (two-way repeated measures ANOVA: condition, $F = 0.081$, $p > 0.05$); there was a significant reduction in blood alcohol levels in both groups of mice during the second exposure to alcohol, relative to the first ($F = 6.542$, $p < 0.01$). **(F)** The effect of chronic sleep restriction was mimicked in mice receiving continuous i.c.v. infusion of DPCPX (100μM), which were less sensitive to the motor-impairing effects of alcohol compared to vehicle (Veh) treated mice (two-way repeated measures ANOVA: condition, $F = 1.749$, $p > 0.05$; time, $F = 39.955$, $p < 0.001$; condition x time, $F = 2.446$, p

< 0.05; Bonferonni post-hoc test, *p < 0.05; **p < 0.01). The n value depicted in this figure indicates the number of animals. This work was performed by Sally McIver.

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80 min post-injection. Performance on the rotarod (measured as latency to fall as a percent baseline) was significantly higher in sleep restricted mice compared to non-sleep restricted mice, (Fig. 4.8 A), suggesting that sleep restricted mice have reduced sensitivity to the motor-impairing effects of alcohol. Given that chronic sleep restriction had a sustained impact on adenosine tone (Fig. 4.5 B) the mice were re-probed for sensitivity to the motor-impairing effects of alcohol 2 and 4 weeks later. Sleep restricted mice were still significantly less impaired than non-sleep restricted mice at each of these time points (Fig. 4.8 B, C). In agreement with these findings, the time to 50% recovery of performance was significantly shorter in sleep restricted mice compared to controls following 24 h and 2 weeks of recovery from sleep restriction (Fig. 8D). Importantly, this reduced sensitivity persisted in the absence of altered blood alcohol levels (Fig. 4.8 E), suggesting that the difference in sensitivity was not due to altered alcohol metabolism, but rather was a central response. Furthermore, chronic intracerebroventricular (i.c.v.) infusion of the A1R antagonist DPCPX produced a similar reduction in sensitivity to the motor-impairing response to alcohol (Fig. 4.8 F), consistent with the notion that attenuated A1R signaling contributes to reduced alcohol sensitivity in mice that have been chronically sleep restricted.

Chapter 5: Astrocytic Calcium Signaling Enhances Low Threshold Potentiation through an Adenosine Mediated Increase in NMDA Receptor Responses

Contributing Authors: Lee, SY. and Hines D. performed experiments included in this chapter. Papouin T., Blutstein T. and Halassa M. were consulted during the design of this research.

Section 5.1: Overview

A variety of studies have demonstrated that astrocytes are capable of reciprocally communicating with neurons (Shelton and McCarthy, 2000; Halassa et al., 2007a; Zorec et al., 2012). One mechanism mediating this interaction lies in the ability of G-protein coupled receptors expressed by astrocytes to respond to neurotransmitters released by neurons via activation of downstream signaling cascades (Agulhon et al., 2012). Activation of these receptors can drive a variety of cellular responses including elevation of cytosolic calcium (Shelton and McCarthy, 2000). This can then cause calcium dependent release of signaling molecules, termed gliotransmitters (Fellin and Carmignoto, 2004; Halassa et al., 2007a; Parpura and Zorec, 2010) which allow astrocytes to modulate synaptic transmission. Gliotransmission can affect both release probability from the pre-synaptic terminal and receptor surface expression within spines and dendrites (Newman, 2003; Deng et al., 2011; Schmitt et al., 2012). Molecular genetic inactivation of vesicular release disrupts this interaction, and this manipulation leads to a variety of effects including a reduction in adorA1 receptor mediated inhibition as well as suppression of synaptic plasticity (Pascual et al., 2005; Fellin et al., 2009; Halassa et al., 2009b). In addition to their canonical inhibitory role, recent results have demonstrated that activation of postsynaptic adorA1 receptors can lead to phosphorylation and reduced internalization of NMDA receptors (Deng et al., 2011). These finding suggests that adenosine may have a dual function at synapses: acting on

presynaptic adorA1 receptors to suppress release while activating postsynaptic receptors to enhance NMDA receptor surface expression, potentially leading to lowered threshold for long term potentiation (Lau and Zukin, 2007). The capacity of adenosine to coordinate both pre- and post- synaptic activity suggests that this transmitter may play a role in coordinating synaptic transmission (Pascual et al., 2005) allowing astrocytes to tune activity and to optimize the signal to noise ratio. For this to be the case, activation of astrocytic calcium in response to synaptic activity or other signals would need to lead to higher extracellular adenosine. Although several studies have suggested that astrocytes provide a major source of extracellular adenosine, most likely through vesicular release of ATP which is rapidly converted to adenosine in the extracellular space (Fields and Burnstock, 2006), an increase in the extracellular concentration of this transmitter in response to calcium elevation has not been shown in acutely isolated tissue. The dynamics of release through this pathway therefore remain unexplored. The details of these dynamics may be critical to the effects of activating this pathway since, depending on the location and concentration of adenosine, it would either reduce glutamate release through presynaptic inhibition of voltage gated calcium channels or enhance the glutamate response relevant to plasticity by increasing the activity of NMDA receptors.

In this study, we investigated the hypothesis that activating astrocyte specific receptors can affect synaptic transmission by controlling extracellular adenosine. We showed that astrocytes respond to agonists for the Gq coupled protease activated receptor type 1 (PAR1) by mobilization of calcium through an IP_3 dependent mechanism. Activating calcium signaling through this pathway leads to a dose dependent inhibition of

excitatory synaptic transmission by increasing the level of extracellular adenosine. In addition, triggering this pathway at a relatively low dose of the agonist causes an enhancement of NMDA receptor mediated synaptic transmission through a mechanism that depends on adorA1 receptor activation. This leads to an enhancement of NMDA receptor dependent LTP allowing relatively weak input to strengthen synapses. Together, these results suggest that astrocytic calcium signaling can tune synaptic interactions through adenosine and suggest that this pathway has a previously un-recognized role in rapid, metaplastic enhancement of synaptic plasticity.

Section 5.2: Specific Methods

Tissue processing and Immunohistochemistry: 8-10 weak old animals were cardiac perfused with phosphate buffered saline (PBS) and subsequently with paraformaldehyde (PFA). The brain was then extracted, post-fixed in PFA for 1 hour at 4 °C, placed overnight in 30% sucrose and finally frozen and stored at -80 °C. Coronal sections (40 μm) were cut, subsequently washed with PBS three times and permeabilized with 0.1% Triton X-100, 5% horse or goat serum in PBS. Unless otherwise indicated the antibodies used were from Millipore, Temecula, CA. We used mouse NeuN antibody, 1:1000, rabbit NG2 antibody, 1:1000, rabbit GFAP antibody (Sigma, St. Louis, MO), 1:1000, rabbit Iba1 antibody (Wako Chemicals, Richmond, VA), 1:500. Secondary antibodies conjugated to either Alexa 633 or 568 were then used. Sections were mounted and visualized on either Fluoview 1000 confocal microscope (Olympus, Center Valley, PA) or A1 confocal microscope (Nikon, Tokyo, Japan). Venus fluorescence was detected at 515 nm.

Slice Preparation for Calcium imaging: Hippocampal slices 400 um thick were cut in artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM Glucose, 1 mM Sodium Pyruvate, 2.9 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) which was kept near freezing by periodic addition of frozen sucrose to the cutting chamber. Slices were then allowed to recover for one hour. Following recovery, slices were pre-incubated with the calcium indicator dye Rhod2 (3 mM) for an additional 1 hour to allow dye loading.

Calcium Imaging and Analysis: Imaging was conducted using a commercially produced upright two-photon microscope (Prairie Technologies) fitted with a slice recording chamber including a continuously heated bath enclosure (Harvard Apparatus, 32.8º C). Slices were placed in a submerged recording chamber and continuously perfused with recording artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM Glucose, 1 mM Sodium Pyruvate, 2.9 mM KCl, 2mM $CaCl_2$, 1 mM $MgCl_2$) at 1.5-2 mL/min. Two-photon imaging was performed by using an Ultima scanhead (Prairie Technologies, Middleton, WI) attached to an Olympus BX51WI microscope equipped with a ´60 water-immersion objective. Excitation for YFP and Rhod-2 was provided at 820 nm, and emission was detected by external photomultiplier tubes (525/70; DLCP 575; 607/45 nm). To account for differences in loading, regions of interest (ROI) were normalized to baseline florescence. The fluorescent signal at a given time point was expressed as DF/F = (F1 - F0)/F0, where F0 and F1 are the value of the fluorescence in the ROI at rest and at the given time point, respectively.

Extracellular field recordings: All recordings of AMPA receptor mediated fEPSP were made in recording artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM Glucose, 1 mM Sodium Pyruvate, 2.9 mM KCl, 2mM $CaCl_2$, 1 mM $MgCl_2$). For NMDA receptor specific fEPSPs aCSF contained lower magnesium to reduce the activity dependent block of this ionotropic receptor (124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM Glucose, 2.9 mM KCl, 2mM $CaCl_2$, 0.2 mM $MgCl_2$). In both sets of recordings, aCSF was bubbled to saturation with a 95/5% mixture of O2/CO2 and continuously superfused at 1.6 mL/minute and maintained at 32.8 °C. Glass recording electrodes filled with aCSF were positioned in area CA1. For NMDA receptor specific fEPSPs, following placement of the electrodes NBQX (4 uM) and Picrotoxin (50 uM) were applied and the signal was allowed at least 40 minutes to stabilize prior to the start of the baseline. Signals were amplified and band pass filtered between 0.1Hz and 1 kHz using a 1800 microelectrode amplifier (A-M Systems) and digitized at 5 kHz via a Digidata 1320 digitzer (Molecular Devices). The CA1-CA2 boundary was severed and paired stimulation pulses were delivered using a 125 µm concentric Pt-Ir electrode in the stratum radiatum to orthodromically stimulate axons entering area CA1 using a paired pulse protocol with an interstimulus interval of 100 ms (sweeps frequency = 0.033 Hz). Recordings were acquired with Clampex 9.2 software and analyzed using Clampfit (Molecular Devices).

Analysis of Adenosine Biosensor Measurements: Biosensor based recordings were performed as described previously (Chapter 2). Estimation of tonic extracellular adenosine was made using INO subtracted ADO signals following the 20 minute

stabilization period. Potentiostat based recordings were made using the ME200+ Duo-Stat (Sycopel International Ltd. Jarrow, UK) and were digitized via a Digidata 1320 digitzer (Molecular Devices). Acquisition utilized Clampex 9.2 software (Molecular Devices). Following acquisition, signal capacitance was corrected for each sensor offline by fitting a single exponential to the portion of the trace prior to the insertion of the sensors into the tissue. This correction was performed individually for each sensor prior to calibration. To isolate the adenosine component, the resulting, capacitance corrected signals were calibrated differentially based on the response of each sensor to adenosine or inosine standards and the INO response component was subtracted from the ADO sensor signal.

Section 5.3: Results

Activating Astrocyte Specific G-protein Coupled Protease Activated Receptor 1 Causes an IP_3 Dependent Calcium Elevation

Astrocytic calcium signaling can be triggered in response to several different signaling cascades including G-protein coupled receptors. One key mechanism by which this occurs is through activation of Gq coupled receptors which increases phospholipase C activity leading to an increase in IP_3 levels within the cytosol (Neves et al., 2002). This IP_3 can subsequently activate InsP3 receptors in the endoplastic reticulum, leading to release of calcium from this intracellular store into the cytoplasm. Astrocytes express a wide variety of Gq coupled receptors some of which are highly enriched in these cells relative to neurons or other cell types within the CNS (Shelton and McCarthy, 2000). Although there is considerable overlap between the expression of these receptors in

neurons and astrocytes, expression profiling has revealed that there are subsets expressed, at least primarily, in neurons or astrocytes alone (Cahoy et al., 2008). One of the receptors within this group, PAR1, has previously been shown to selectively activate calcium signaling in astrocytes but not neurons *in vitro* and *in situ* providing a means to selectively stimulate astrocytes while minimally impacting neurons (Lee et al., 2007). Because this receptor is known to couple to more than one downstream pathway (McCoy et al., 2012), however, previous studies had not conclusively determined the mechanism mediating the astrocytic calcium response it produces.

To determine whether the astrocyte specific calcium response mediated by PAR1 receptors required IP_3 dependent mobilization of intracellular calcium, we made use of a transgenic mouse model in which this signaling pathway is disrupted specifically in astrocytes. In these mice, a fusion protein containing IP_3 phosphatase tagged with venus yellow fluorescent protein (VIPP) is conditionally expressed using tetO under control of the astrocyte specific GFAP promoter (Fig. 5.1 and 5.2 A). This enzyme blocks IP_3 signaling by dephosphorylating IP_3 to IP_2 thereby interrupting Gq coupled signaling mediated by activation of PLC. Because the dephosphorylated form of inositol does not activate InsP3Rs, expressing this enzyme at high levels prevents activation of this receptor and thus inhibits the liberation of calcium from the ER in response to activation of this pathway. Although this form of calcium signaling is critical for a variety of astrocytic functions during development, the tet "Off" driver allows transcription of the VIPP transgene to be suppressed by doxycycline, which is provided throughout early development to obviate any possible developmental effects of interfering with this pathway. Double-labeling experiments demonstrated that VIPP was selectively

Figure 5.1

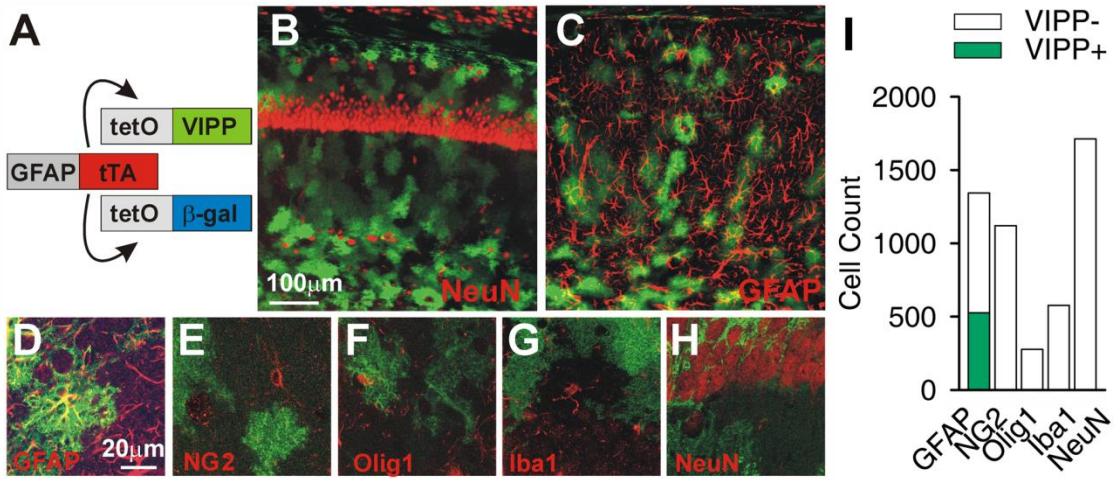

Figure 5.1: Conditional, astrocyte-specific expression of VIPP hippocampal area CA1. (A) GFAP promoter driving the expression of VIPP and β-gal in bigenic VIPP animals. **(B-C)** Top view confocal images (20x 0.7NA objective) of hippocampal CA1-region showing VIPP expression and CA1 neurons (NeuN) and astrocytes (GFAP). **(D-H)** High magnification single confocal planes (60x 1.4NA objective) showing VIPP colocalizes with GFAP (D), but not with NG2 (E), Olig1 (F) Iba1 (G) or NeuN (H). **(I)** Quantification of D-H. n = 6 animals for each staining. This work was performed by Michael Halassa and So-Young Lee.

expressed in astrocytes. Venus fluorescence was observed in 39% of hippocampal area CA1 GFAP positive astrocytes (n = 527 out of 1343 from 6 animals; Fig. 5.1 C, D, I), but was never detected in neurons (NeuN; Fig. 5.1 B, H), NG2-glia (NG2; Fig. 5.1 E), oligodendrocytes (olig1; Fig. 5.1 F) or microglia (Iba1; Fig. 5.1 G).

Using this mouse model, we performed calcium imaging within the hippocampus by first incubating slices in Rhod2, a calcium sensitive dye that is preferentially taken up by astrocytes. Following loading, colocalization was observed between this indicator and YFP in cells with protoplasmic glia-like morphology suggested that this dye was present within VIPP expressing astrocytes (Fig. 5.2 B). Under physiological conditions, PAR1 is activated following cleavage of the extracellular portion of the C-terminal by serine proteases such as thrombin. This cleavage removes a large portion of the protein and results in a chained peptide that can bind to other parts of the receptor, causing a conformational shift that leads to autoactivation (Vu et al., 1991). Because this activation does not allow washout, however, we instead used a peptide agonist, TFLLR. This peptide is structurally similar to the terminal fragment produced by proteases and is sufficient to activate the receptor without cleavage (McCoy et al., 2012). This allowed us to selectively activate PAR1 without permanently modifying the protein. Using this approach therefore allowed us to transiently activate PAR1 and then to terminate this activation by washing out the peptide. Application of TFLLR produced a dramatic rise in calcium signaling within astrocytes in WT slices, suggesting that this peptide was sufficient to activate the receptor and liberate cytosolic calcium. In VIPP expressing cells, however, this rise was markedly reduced (Fig. 5.2C). Quantification of the total

Figure 5.2:

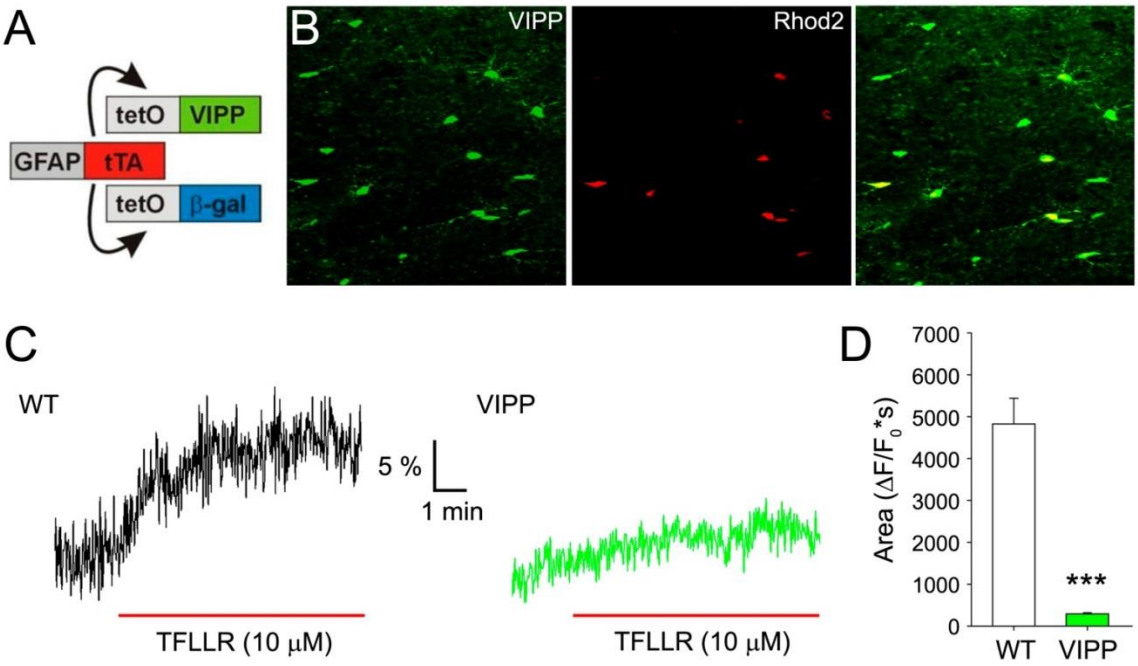

Figure 5.2: PAR1 agonists induce astrocytic calcium through an IP_3 dependent mechanism. **(A)** Schematic diagram showing the expression system used to drive VIPP expression allowing conditional astrocyte specific expression of this transgene along with Beta-galactosidase under the tetO promoter. **(B)** Example of Rhod2 loading in a VIPP expressing slices showing VIPP fluorescence (left panel), Rhod2 fluorescence (middle panel) and overlay (right panel). **(C)** Average trace showing response to applications of 10 uM TFLLR in WT (left panel, n = 5) or VIPP (right panel, n = 5). **(D)** Quantification of the integrated fluorescence signal normalized to baseline loading to control for variation in this parameter (*** $p < 0.005$ Bonferroni Single Comparison Test). This work was performed by Dustin Hines.

fluorescence signal showed a significant reduction in the response of VIPP expressing astrocytes relative to those in control slices. These findings demonstrate that expression of this transgene significantly abrogated the astrocytic calcium response to TFLLR, suggesting that PAR1 activation of calcium signaling in these cells depends on IP_3 mediated liberation of cytosolic calcium (Fig. 5.2D).

Activating IP_3 dependent calcium mobilization produces a dose dependent increase in astrocyte-derived adenosine

Elevation of cytosolic calcium through G-protein coupled receptors and other mechanisms is thought to provide the primary mechanism by which astrocytes respond to neuronal or astrocytic signals. Increased cytosolic calcium triggered through this pathway allows these cells to release transmitters through multiple, calcium-dependent pathways (Zorec et al., 2012). This response is widely thought to provide the means by which astrocytes can reciprocally communicate with neurons and other astrocytes (Halassa et al., 2007a). Adenosine is among the key astrocyte-derived signaling molecules and levels of this gliotransmitter are reduced by disruption vesicular release (Pascual et al., 2005; Halassa et al., 2009b; Schmitt et al., 2012). Although astrocytes are not thought to release adenosine directly through a vesicular mechanism, under some conditions they do release ATP through calcium dependent vesicular fusion (Pangrsic et al., 2007). Since this ATP can be rapidly hydrolyzed to adenosine in the extracellular space (Dunwiddie et al., 1997), this provides a potential calcium dependent source of adenosine.

To investigate whether activation of astrocytic calcium is sufficient to elevate extracellular adenosine through a SNARE dependent mechanism, we used enzyme

assisted, amperometric biosensors to directly measure extracellular adenosine in area CA1 within the hippocampus. We then applied the PAR1 agonist TFLLR to activate IP_3 dependent astrocytic calcium signaling. Superfusion of this agonist at a concentration sufficient to produce a calcium response produced an increase in extracellular adenosine over a time scale comparable to that of the calcium elevation (Fig. 5.3A, top inset). Higher concentrations of TFLLR caused correspondingly greater increases in the level of adenosine release. Interestingly, this dose dependence was relatively non-linear with a three-fold increase in agonist concentration eliciting a ten-fold greater adenosine response (Fig.5.3A). Although the basis of this non-linear response is unclear, one possibility is that at relatively high levels of release ATP activates astrocytic purinergic receptors. Activation of this receptor is known to induce calcium activity in astrocytes (Hashioka et al., 2014) and thus could produce a feed-forward increase in calcium dependent release by these cells.

We next tested whether the observed adenosine response to PAR1 activation required astrocytic IP_3 dependent calcium signals and whether it was mediated by SNARE dependent vesicular release. The response to TFLLR in VIPP slices was significantly reduced relative to WT controls (Fig. 5.3B) suggesting that adenosine signaling did depend on IP_3 mediated liberation of calcium. In addition, astrocyte specific expression of the dnSNARE transgene to disrupt vesicular release also significantly reduced the elevation of adenosine (Fig. 5.3B). Although not conclusive, this suggests that the increase in astrocyte-derived adenosine depends on vesicular release, most likely of ATP. Regardless of the precise mechanism, however, these findings show that astrocytes can increase the level of extracellular adenosine through a

Figure 5.3:

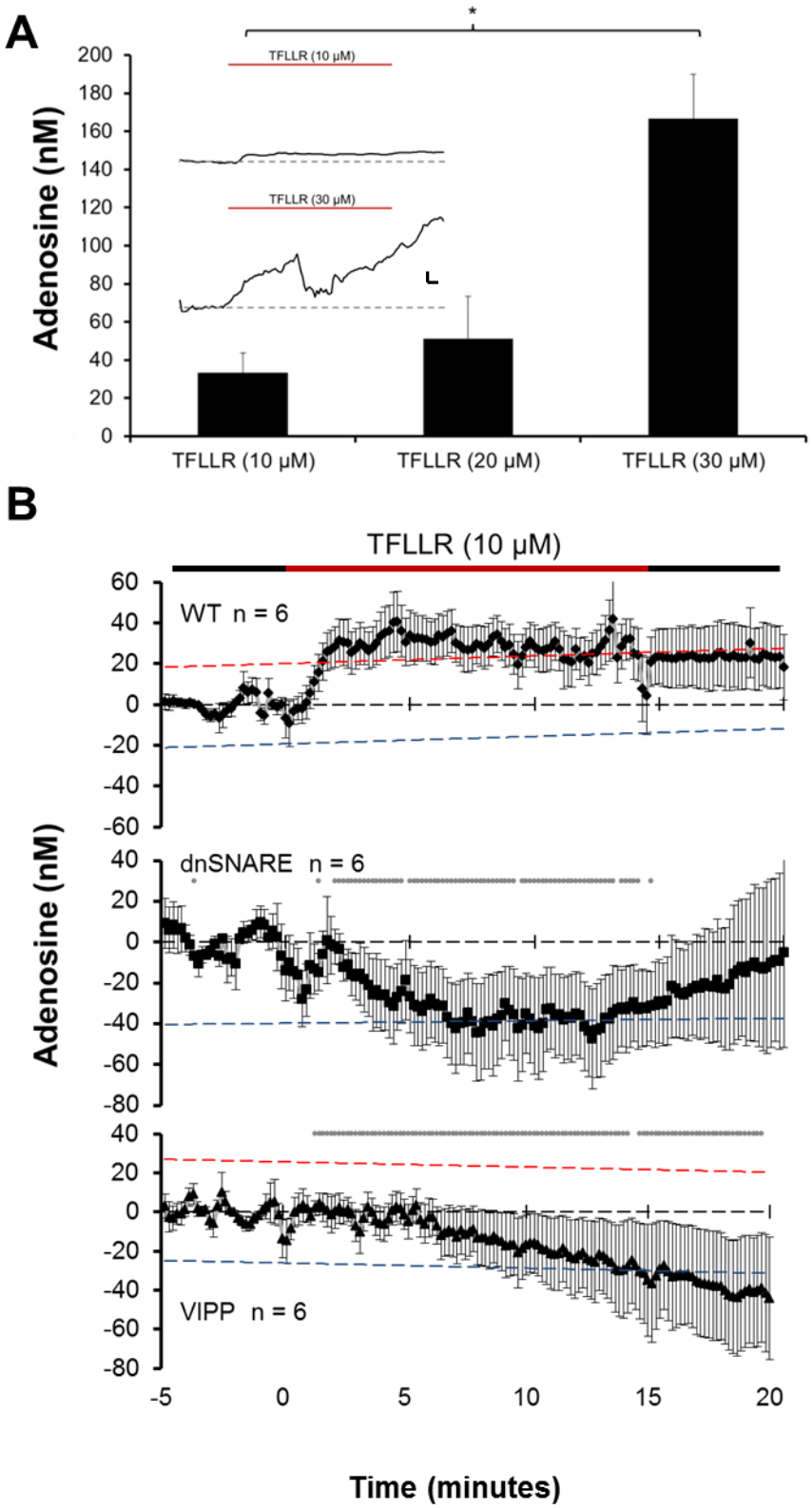

Figure 5.3: Astrocytic IP_3 signaling causes a SNARE mediated elevation of astrocyte-derived adenosine. **(A)** Transient application of TFLLR to wt slices increased

extracellular adenosine with greater increases observed in response to higher concentrations of the peptide (SNK-t test with bonferonin correction *$p < 0.05$, scale bar 50 nM vertical 1 minute horizontal). Inset: Example traces showing the change in extracellular adenosine following application of TFLLR. **(B)** The increase in extracellular adenosine in response to TFLLR was significantly abrogated by expression of either the dnSNARE or VIPP transgenes, indicating that the increase required both normal SNARE function and IP_3 dependent release of calcium in astrocytes (2-way RM ANOVA (Time x Genotype interaction) $p < 0.001$ SNK–t test vs WT * $p < 0.05$)

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mechanism that depends on IP_3 induced calcium signaling and SNARE function within astrocytes.

Astrocyte-Derived Adenosine Produces Distinct Responses Depending on the Level of Activation by G-protein Coupled Receptors

The canonical pathway by which extracellular adenosine modulates synaptic transmission is through a combination of presynaptic inhibition as well as somatic activation of GIRK potassium currents, leading to hyperpolarization of neurons (Trussell and Jackson, 1985). Both of these responses are mediated by adorA1 receptors through a Gi coupled signaling pathway (Cordeaux et al., 2004). We therefore attempted to determine whether astrocyte-derived adenosine release through this mechanism was able to inhibit synaptic transmission. We focused specifically on synapses of the schaefer collateral projection to hippocampal area CA1 because this area is known to have a tonic level of adenosine mediated inhibition which depends on astrocytic release (Pascual et al., 2005; Halassa et al., 2009b). Although application of 10 μM TFLLR was sufficient to both elevate astrocytic calcium (Fig. 5.2 B) and produce a measureable increase in extracellular adenosine (Fig. 5.3 A-B) activation of PAR1 receptors with this concentration of the agonist did not produce any apparent inhibitory effect (Fig. 5.4A, top

Figure 5.4:

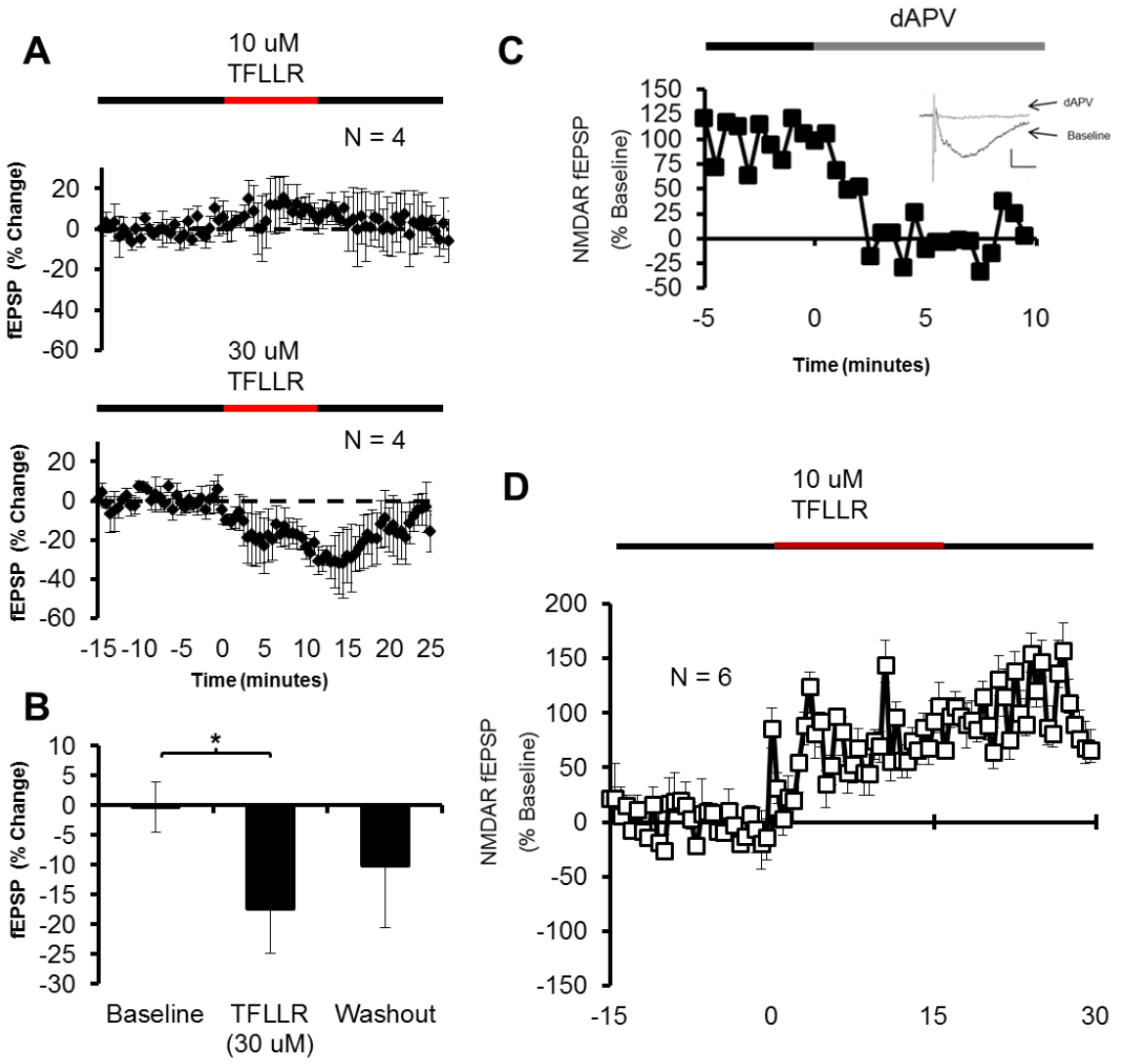

Figure 5.4: Dose dependent control of astrocyte-derived adenosine differentially effects pre- and post- synaptic transmission. **(A)** Average response to application of TFLLR at 10 uM or 30 uM. A suppression is observed only at the higher concentration of the agonist. **(B)** Application of 30 uM TFLLR produces a significant inhibition which is no longer present during washout (ANOVA $p < 0.05$, SNK-t test vs Baseline, * $p < 0.05$). **(C)** Example of a NMDA-fEPSP recording showing typical field responses (inset). These responses are completely blocked by d-APV indicating that they are mediated solely by NMDA receptors. **(D)** Application of TFLLR dramatically enhances NMDA-fEPSPs

panel). Interestingly, however, at higher concentrations of TFLLR the fEPSP slope was significantly suppressed. This suggests the possibility that astrocytic release of adenosine may occur preferentially away from the presynaptic terminal, since biosensor based measurement is more sensitive to adenosine in this portion of the slice. It also indicates, however, that at a sufficient level of activation adenosine released in response to G-protein dependent calcium signaling is capable of producing synaptic inhibition (Fig. 5.4A-B).

Because activation of postsynaptic adorA1Rs has been shown to increase NMDAR mediated currents through src dependent stabilization of the receptor on the synaptic membrane (Deng et al., 2011), we next tested the effect of calcium dependent astrocyte-derived adenosine on these receptors. To accomplish this we measured the effect of TFLLR on NMDAR mediated field extracellular potentials (NMDAR-fEPSPs). To isolate the potential change produced by glutamate acting on this receptor, we stimulated schaeffer collateral fibers and recorded in low (0.2 mM) magnesium aCSF in the presence of the AMPAR antagonist NBQX (4 uM). The field response showed the characteristic slow kinetics associated with NMDA receptor activation and the response was completely blocked by the NMDAR antagonist d-APV (Fig. 5.4C). Unexpectedly, low concentrations of TFLLR (10 uM), which had no effect on presynaptic inhibition, produced a dramatic enhancement of the NMDAR-fEPSP slope (Fig. 5.4 D).

The apparent dose dependence of the pre- versus post- synaptic effects of PAR1 activation allowed us to use a lower concentration of the agonist to specifically dissect the effects of this pathway on NMDA receptors. Given that blocking SNARE dependent vesicular fusion by expression of dnSNARE was sufficient to prevent the adenosine

Figure 5.5:

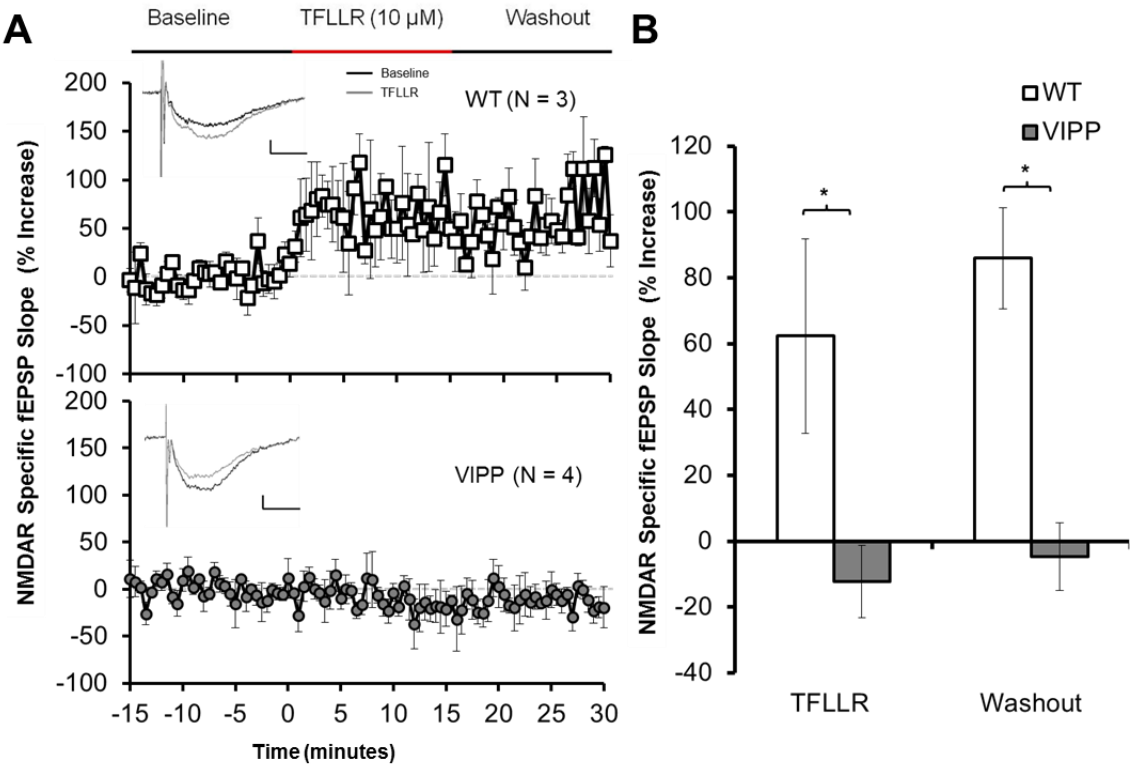

Figure 5.5: Application of TFLLR increases NMDAR-fEPSP strength through astrocytic IP_3 dependent signaling. **(A)** Transient application of TFLLR produced a large and long lasting increase in fEPSP slope recorded in aCSF containing low magnesium (0.2 mM) and NBQX (4µM) to isolate NMDA mediated potentials. This increase was prevented by expression of VIPP suggestion that it required astrocytic IP_3 signaling. **(B)** Expression of the VIPP transgene significantly abrogates the increase in NMDAR-fEPSP by TFLLR averaged over the last five minutes of application (TFLLR) and following ten minutes of washout (Washout). (2-way ANOVA $p < 0.01$, SNK t-test, * $p < 0.05$)

response to TFLLR, we sought to test whether expression of this transgene was sufficient to block PAR1 dependent enhancement of NMDAR-fEPSPs. We found that while activation of this receptor increased fEPSP response slopes (76.75 +/- 50.5 %) in dnSNARE slices this effect was significantly abrogated and, in fact, these slices showed a slight inhibition in response to TFLLR (-57.98 +/- 11.7%, $p < 0.05$ SNK t-test comparison). This suggests that the effect of PAR1 activation requires normal SNARE function but it does not speak to the involvement of the astrocytic calcium response produced by this receptor.

IP_3 Dependent Astrocytic Calcium Activity Leads to Enhancement of NMDAR-fEPSPs mediated by adorA1R activation

Because some previous studies have suggested that protease activated receptors can be expressed on neurons as well as glia (Gingrich et al., 2000) it remained possible that the effects of TFLLR on NMDAR-fEPSPs occurred through regulation of neurons, either directly by acting on post-synaptic PAR1 receptors or indirectly by modulating GABAergic inhibition. To address these possibilities, we recorded NMDAR-fEPSPs in the presence of picrotoxin to block $GABA_A$ mediated inhibition. We found that the effect of TFLLR was preserved under these recording conditions (Fig. 5.5 A), suggesting that disruption of inhibition was not responsible for the observed increase in NMDAR-fEPSP slope.

We next investigated whether the observed potentiation of NMDAR-fEPSPs was due to direct activation of neuronal receptors or whether it instead required astrocytic calcium signaling via the InsP3R activation. The effect of TFLLR at 10 uM on NMDAR-fEPSPs

Figure 5.6:

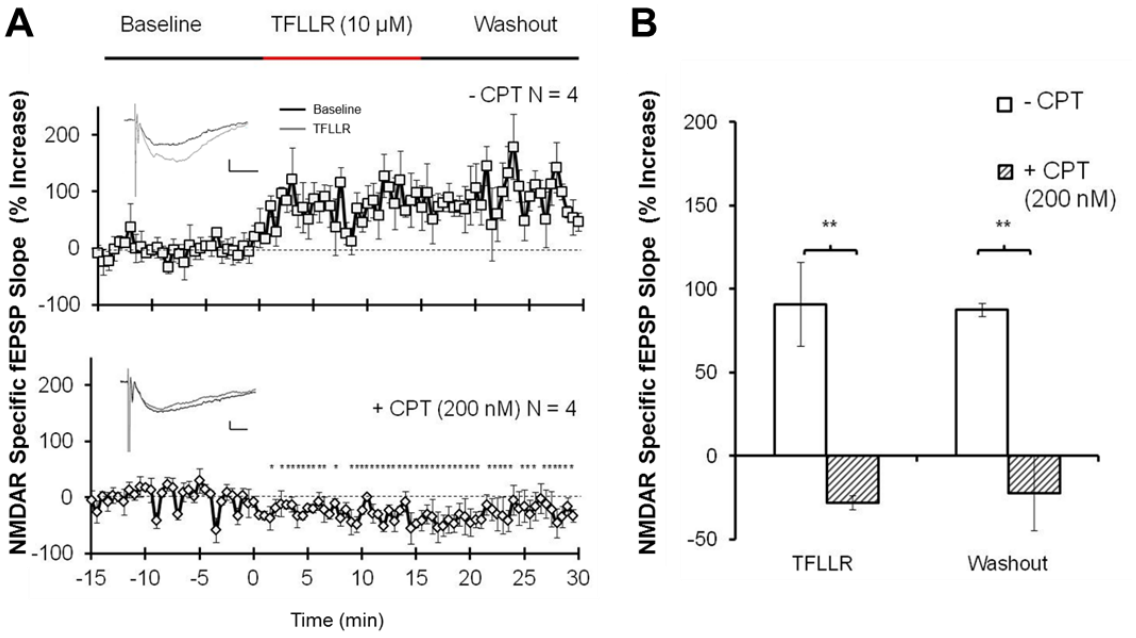

Figure 5.6: Activation of astrocytic calcium signaling potentiates NMDAR activity through an adenosine mediated mechanism. **(A)** Application of TFLLR produced a sustained rise in NMDA specific fEPSP slope however this was blocked in the presence of CPT (200 nM, $p < 0.005$ two way RM-ANOVA, * $p < 0.05$ SNK t-test) **(B)** Following application of TFLLR, the peak NMDAR mediated fEPSP was significantly enhanced in the absence of CPT relative to those in which CPT was present. (SNK t-test with Bonferroni Correction, ** $p < 0.01$).

was significantly reduced in slices taken from VIPP mice (Fig. 5.5 A-B), suggesting that astrocytes were, in fact, responsible for the effect of PAR1 activation. This finding also indicates that the pathway leading to enhancement of NMDAR-fEPSPs through activation of this receptor was IP_3 dependent, implying that it involves calcium dependent release.

Previous studies have demonstrated that activation of adorA1 receptors is sufficient to phosphorylate NMDA subunits leading to increased surface levels of this receptor (Deng et al., 2011). Because we observe a rise in adenosine following activation of astrocytic calcium signaling, one possible explanation for the observed enhancement of NMDAR-fEPSPs is that it is produced by adenosine acting on post-synaptic adorA1 receptors. To determine whether this receptor is involved, we applied TFLLR in the presences of the adorA1 receptor antagonists CPT (200 nM). Following stabilization of the signal, CPT was applied for thirty minutes prior to the start of the baseline. Because the recording conditions used for this experiment include high influx of calcium through NMDA receptors and blockade of ionotropic GABA receptors, extended recordings of NMDAR-fEPSPs can lead to hyperexcitability. To exclude the possibility that longer recording times needed to apply CPT were involved in modifying the potentiation in response to PAR1 activation, control slices for this experiment were also maintained for 30 minutes following stabilization of the baseline even though CPT was not applied. Despite this control, the effect of activating astrocytic calcium signaling with TFLLR was still evident in control slices however it was significantly reduced by CPT suggesting that this effect was mediated by activation of adorA1 receptors (Fig. 5.6A-B).

Activating astrocytic calcium produces a metaplastic enhancement of low threshold plasticity via adorA1 receptor activation

It is well established that NMDA receptors play a key role in certain forms of synaptic plasticity in the hippocampus as well as other parts of the brain (Abel et al., 1997; Nguyen and Kandel, 1997; Malenka and Bear, 2004). Given our observation that astrocytes can enhance NMDAR activity through adenosine signaling we next tested whether this had any metaplastic impact on LTP. Theta burst stimulation of the Schaefer collateral synapse was used to provide a physiologically relevant version of LTP which is known to be NMDAR dependent. We used a low intensity, "threshold", LTP induction protocol in which two trains of theta burst (2x theta) stimulation were used rather than the stronger three to five theta burst protocol which is frequently employed for LTP induction (Reyes and Stanton, 1996; Nguyen and Kandel, 1997). Bath application of TFLLR for ten minutes prior to theta burst stimulation using this protocol markedly enhanced the potentiation by 2xtheta stimulation (Fig. 5.7A). Pre-application of TFLLR significantly increased LTP but this effect was absent in VIPP slices (Fig. 5.7A-B) suggesting that the enhancement required astrocytic InsP3R mediated calcium signaling. Interestingly, the effect was also absent for stronger induction protocols (Fig. 5.7C) suggesting that this form of metaplaticity lowered the threshold for higher level potentiation but did not play a role in "saturating" LTP induction.

Astrocytes release a variety of transmitters which can affect NMDA receptors through direct and indirect mechanisms. These include glutamate, which is known to modulate postsynaptic receptors through activation of metabotropic glutamate receptors (Halassa and Haydon, 2010; Pirttimaki et al., 2011). In addition, they are known to

Figure 5.7:

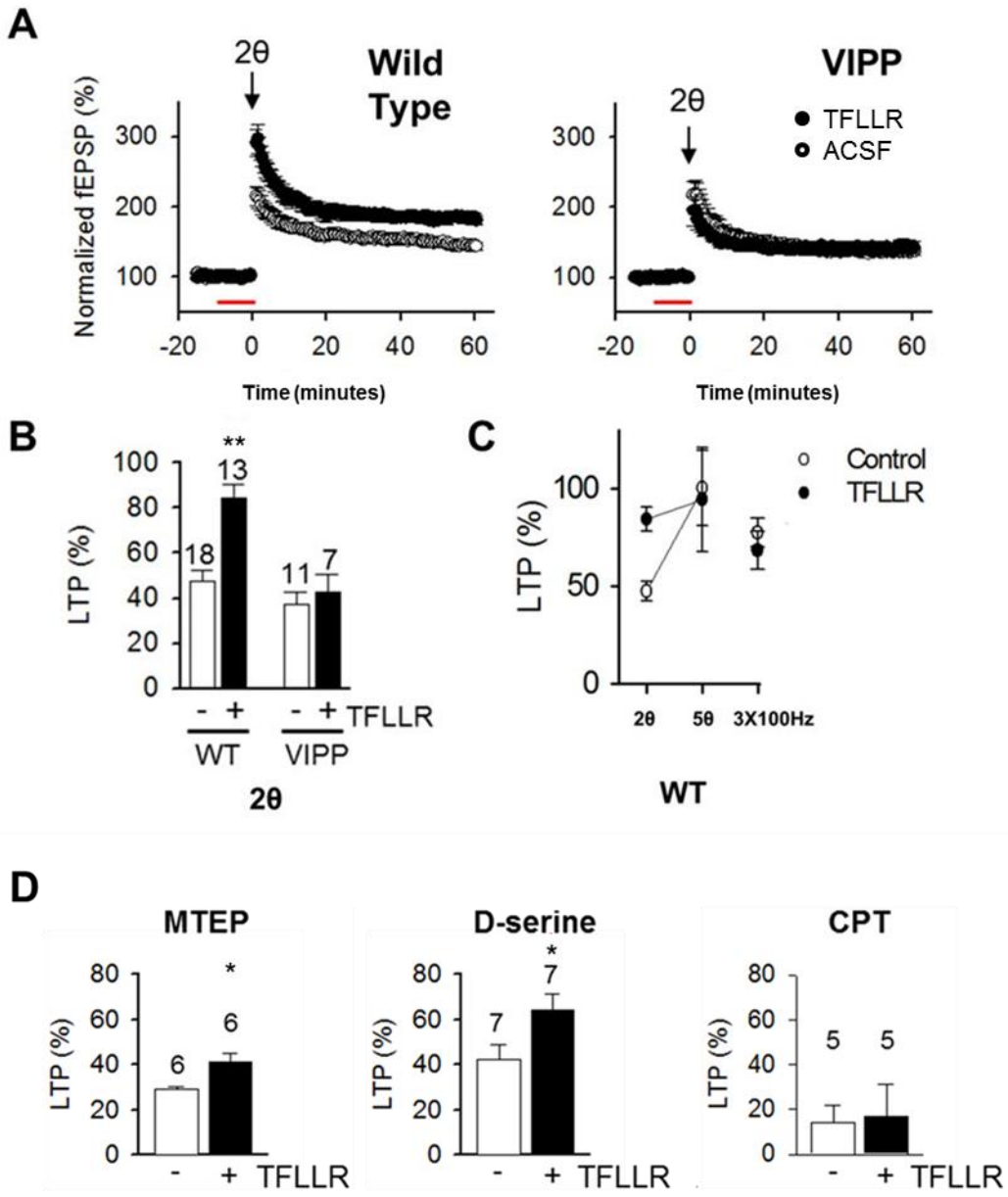

Figure 5.7: Activation of astrocytic IP_3 signaling causes an adenosine A1 receptor dependent enhancement of low-threshold LTP. **(A)** Normal aCSF (empty circles) or 10 μM of the PAR1 receptor agonist TFLLR (filled circles) was applied prior to induction of LTP (red bar) via a low-threshold protocol consisting of two trains of theta bursts. **(B)** Pre-treatement with TFLLR enhanced this form of LTP in Wild Type slices but this enhancement was blocked by expression of VIPP (** $p < 0.01$ SNK t-test). **(C)** The PAR1 dependent enhancement occurred in low threshold LTP but not in higher intensity induction protocols. **(D)** Neither the mGluR5 receptor antagonist MTEP nor application of excess D-serine prevented the effect of TFLLR on low threshold LTP however the

effect was blocked by the A1 receptor antagonist CPT (* $p < 0.05$ SNK t-test). This work was performed by So-Young Lee.

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release D-serine, which can also be released from astrocytes, which acts as an endogenous coagonist of NMDARs(Henneberger et al., 2010; Martineau et al., 2013). Because of this, we next tested whether these gliotransmitters might play a role in the metaplastic effect of TFLLR or whether it was instead due to activation of adorA1 receptors by astrocyte-derived adenosine. Neither the metabotropic glutamate receptor inhibitor 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP) nor application of exogenous D-serine prevented the enhancement of 2xtheta induced LTP by TFLLR however CPT blocked this effect (Fig. 5.7D-F). Together these findings support the hypothesis that activation of IP_3 dependent calcium signaling by this agonist enhances low threshold LTP via activation of adorA1 receptors. Based on our earlier observations (Fig. 5.5-5.6 this enhancement is likely to be mediated by post-synaptic augmentation of NMDA receptor activity.

Chapter 6: Discussion and Conclusions

Section 6.1: Overview

Astrocytes are no longer simply the "glue" of the brain. Rather than merely providing the interstitial binding that isolates synapses and maintains the environment for neurons, it is increasingly clear that these cells play an active role in information processing (Fellin and Carmignoto, 2004; Halassa and Haydon, 2010; Perea and Araque, 2010). They have a variety of means with which to accomplish this. Astrocytes control the clearance of many neurotransmitters such as glutamate, allowing them to modulate the rate-constants of neurotransmission. Controlling clearance also allows them the means to govern the level of glutamate and other transmitters which escape the synaptic cleft, thus controlling which receptors are activated (Danbolt, 2001; Nam et al., 2012). Astrocytes also regulate neuronal energy levels through control of the availability of lactate and other metabolic fuels. Availability of these metabolites affects activity and plays an important role in memory function (Suzuki et al., 2011). Perhaps most importantly, astrocytes can respond to incoming signals just as neurons do: by integrating the incoming information, processing it and actively releasing transmitter molecules to carry it forward (Fellin and Carmignoto, 2004; Zhang and Haydon, 2005; Halassa and Haydon, 2010; Parpura and Zorec, 2010). As in neurons, this release is mediated through a variety of mechanisms, the most prominent of which is probably SNARE dependent vesicular exocytosis (Parpura et al., 1994b, 2012; Newman, 2003). Unlike neurons, however, astrocytes are both small and electrically in-excitable so they process information over a very different spatial and temporal domain (Fellin and Carmignoto,

2004; Panatier et al., 2011; Parpura et al., 2012). This distinction is likely to critically determine the roles of these cells in the CNS. It is therefore essential to understand *when* and *where* astrocytic regulation occurs in order to determine the involvement of astrocytes in health and disease.

The research presented in this dissertation attempts to address these questions for one function of astrocytes: the regulation of brain activity in response to wakefulness. In pursuit of this goal, I focused on the effects of astrocytic signaling within the hippocampus, a structure known to be critically associated with declarative memory (Bliss and Collingridge, 1993). Previous findings have strongly implicated extracellular adenosine, which astrocytes can supply through release of ATP and clear through concentration dependent reuptake, in the homeostatic response to wakefulness and in the deficits in hippocampal memory that accompany sleep loss (Bjorness et al., 2009; Elmenhorst et al., 2009; Halassa et al., 2009a; Porkka-Heiskanen and Kalinchuk, 2011; Ben Achour and Pascual, 2012). I have therefore sought to determine whether astrocyte-derived adenosine can be elicited by wakefulness and to understand the consequences of changes in the concentration of this signaling molecule for synaptic transmission and plasticity in the hippocampus.

Using the molecular genetic tools developed in the Haydon lab to selectively disrupt vesicular release (Pascual et al., 2005) and calcium signaling, I have investigated the effect of short term and long term sleep disruption on astrocyte-derived adenosine within the hippocampus as well as the cortex (Chapter 3). My findings demonstrate that acute sleep deprivation increases extracellular adenosine in the hippocampus and activation of inhibitory adenosine receptors in the hippocampus and cortex. This increase

was prevented by dominant negative disruption of SNARE function in astrocytes, suggesting that it was based on a mechanism dependent on vesicular release from these cells. These observations provide clear evidence that astrocytes can respond to wakefulness and that their response to relatively short periods of wakefulness produces a higher level of adenosine within multiple parts of the CNS.

In contrast to the effect of acute deprivation, protracted sleep restriction leads to a reduction in the adenosine response to wakefulness (Chapter 4). The lower adenosine was not due to either lower adenosine receptor surface expression or increased passive clearance suggesting that the reduced adenosine tone was due to a disruption of release. Consistent with this reduction, the homeostatic sleep response is mitigated following sleep restriction suggesting that this allostatic adjustment by astrocytes plays a role in compensating for conditions of chronic sleep disruption. These effects lasted for weeks following the end of sleep restriction suggesting that lower homeostatic elevation of adenosine may contribute to the long term cognitive effects associated with chronic sleep loss (Van Dongen et al., 2003; Banks and Dinges, 2007). An additional consequence of this reduced adenosine is a lower behavioral sensitivity to alcohol. Because higher tolerance is associated with alcohol dependence (Weiss and Porrino, 2002), this response is potentially maladaptive suggesting a mechanism by which sleep problems could contribute to this form of substance abuse.

Because a major means by which astrocytes respond to neurotransmission is through G-protein coupled mobilization of intracellular calcium, I tested the effect of acutely activating this pathway to determine whether it was able to produce an adenosine mediated effect on synaptic transmission (Chapter 5). To do this I activated the astrocyte

enriched PAR1 receptor which is known to increase cytosolic calcium in these cells (Lee et al., 2007; Han et al., 2013). To isolate the astrocytic InsP3R receptor dependent effects of this manipulation on synaptic transmission, I compared the consequences of applying this agonist to WT slices with effects on slices from a mouse model in which IP_3 signaling was blocked by overexpression of IP_3 phosphatase. Using this approach, I determined that G-protein coupled mobilization of astrocytic calcium can lead to an increase in the level of extracellular adenosine. This adenosine affected presynaptic release probability and also modulated synaptic plasticity by increasing NMDAR activity in the post-synaptic terminal. Interestingly, however, the impact of this elevated adenosine depended strongly on the extent to which astrocytic calcium was driven and on the localization of the adenosine receptors involved. In addition, adenosine release was highly nonlinear as a function of G-protein coupled receptor activation suggesting that signaling through this molecular pathway is not as simple as previously believed.

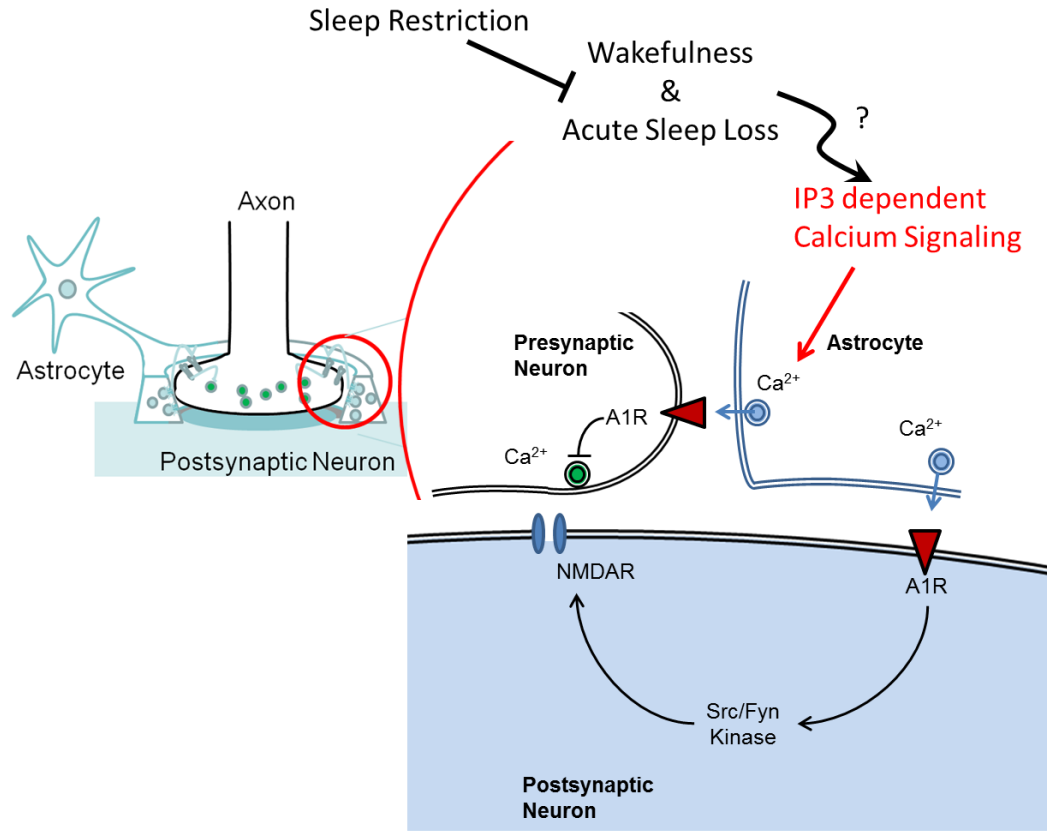
The observation that the threshold for triggering postsynaptic signaling by astrocyte derived adenosine is lower than the threshold for the presynaptic and somatic effects typically ascribed to adenosine receptors is potentially surprising. Our results show that a relatively low level of Gq coupled receptor agonist, while sufficient to drive astrocytic calcium elevation and increases in extracellular adenosine, failed to produce inhibition through adorA1 recepotrs. In contrast, the same concentration of agonist led to a dramatic increase in NMDA receptor mediated field potential, probably by stabilizing this receptor through postsynaptic adorA1R activation, an effect previously demonstrated by Deng and colleagues (Deng et al., 2011). These findings suggest the intriguing possibility that adenosine modulation may be compartmentalized, leading to different

effects depending on the region and/or the amount of adenosine release. The differential effects of adenosine signaling on synaptic transmission provide novel insights into the impact of wakefulness-dependent changes in astrocyte derived adenosine for brain function.

Based on the observations detailed in this document, I propose a model in which astrocytes integrate information based on neuronal activity during wakefulness leading to calcium dependent vesicular release of ATP. Once released into the extracellular space, this ATP is hydrolyzed to adenosine which then acts on presynaptic and/or postsynaptic adorA1 receptors causing inhibition of glutamate release or phosphorylation and reduced internalization of NMDA receptors, respectively (Fig. 6.1). The impact of these changes on hippocampal function remains to be fully characterized, but one consequence is a lower threshold for weak stimuli to induce synaptic plasticity. While this transient elevation of adenosine enhances synaptic plasticity, previous results indicate that prolonged activation of this pathway leads to deficits in hippocampal function (Florian et al., 2011). Perhaps to prevent these deficits, protracted sleep restriction leads to a reduction in the wakefulness-dependent elevation of adenosine along with a corresponding deficit in the homeostatic sleep response.

The findings detailed above suggest that astrocytes are involved in hippocampal function, affecting synaptic transmission in this structure through a wakefulness dependent mechanism. Many questions remain to be answered, however, regarding the details of this response. Further research will be needed to completely characterize both the mechanism by which short term wakefulness triggers the elevation of adenosine

Figure 6.1: Model of Wakefulness Dependent, Calcium Mediated Adenosine Signaling by Astrocytes



through this pathway and to understand the complete consequences of this mechanism for brain function. Some possibilities are discussed in the following sections along with caveats concerning the methods that we have employed. I also discuss the relationship of these observations to previous results and the implication of my findings for the role of astrocytes in healthy brain function and disease. Finally, I conclude with a discussion of experiments suggested by my findings which would contribute to fully characterizing the role of astrocyte-derived adenosine on hippocampal function under different behavioral states.

Section 6.2: Integration of Wakefulness History by Astrocyte-Derived Adenosine

The results detailed in the third chapter of this dissertation demonstrate that wakefulness modulates excitatory synaptic transmission by affecting extracellular adenosine derived from an astrocytic source. These results are potentially surprising given that prior microdialysis studies have not measured an effect of sleep deprivation on extracellular adenosine in hippocampus (Zeitzer et al., 2006). It is important to note, however, that microdialysis only captures a proportion of extracellular adenosine. This coupled with the inherent temporal averaging of *in vivo* microdialysis may limit the capacity of this technique to detect wakefulness-dependent changes in adenosine (Strecker et al., 2006; Kalinchuk et al., 2011).

Throughout the research described in this dissertation, I employ a variety of methods to estimate the level of extracellular adenosine in the central nervous system, several of which utilize the acute hippocampal slice preparation. While my findings

indicate that this preparation provides a consistent estimate of the level of extracellular adenosine based on both receptor activation and direct concentration measurements, a key caveat to this approach is the use of acutely isolated tissue. Many changes occur following removal of the brain and preparation of the hippocampal slice, and these changes can potentially introduce confounds such as hypoxia induced adenosine release (Dale et al., 2000). Although previous studies have demonstrated that incubation of the slice prior to recording can improve its metabolic state and restore energetic markers, including ATP, to levels comparable to those observed in the intact organism (zur Nedden et al., 2011b), it is clear that preparation of the hippocampal slice damages tissue and can affect transmitter release.

In order to address some of the possible concerns with the use of slice preparations, I also performed an *in vivo* experiment showing that wakefulness-dependent modulation of cortical slow oscillations requires an astrocytic source of adenosine. This finding demonstrates that activation of adorA1 receptors is increased by wakefulness within the cortex through an astrocyte dependent mechanism. Although this increase occurs in a different brain region, the pattern is consistent with the wakefulness-dependent elevation measured *in situ* in the hippocampal slice. Together, these observations strongly suggest that wakefulness biases the equilibrium between release and re-uptake of adenosine and that this change is maintained in the hippocampal slice.

Although I demonstrate the importance of a glial source of adenosine for wakefulness-dependent control of extracellular adenosine, my results do not identify the mechanism of these daily changes in adenosine. There are many sites that have the potential to regulate extracellular adenosine including wakefulness-dependent changes in

release, uptake or metabolism of adenosine. In addition, circadian changes in ATP release independent of wakefulness history have been observed in some preparations and this may contribute to the variation of adenosine across the sleep wake cycle (Marpegan et al., 2011), although it is unlikely to be responsible for the increase in adenosine following sleep deprivation. While the observation that ADK dependent clearance is higher following spontaneous wakefulness than it is following the corresponding period of sleep argues against the involvement of this form of clearance in in the higher level of adenosine observed following waking, other mechanisms may still be important. The locus of this regulation therefore requires further investigation.

The level of tonic adenosine that I measured using *in situ* biosensors is higher than previous estimates of average adenosine concentration in the intact rat hippocampus (Carswell et al., 1997) as well as estimates (Dunwiddie and Diao, 1994) and direct measurements (Frenguelli et al., 2007; zur Nedden et al., 2011) made in rat hippocampal slice. Extrapolations of extracellular adenosine concentration in the hippocampal slice based on pharmacological blockade initially estimated a range of 140-200 nM extracellular adenosine acting on synaptic adorA1 receptors in this preparation. Subsequent direct measurements using inserted biosensors in the rat hippocampus also measured tonic adenosine in this range (Frenguelli et al., 2007) and showed a comparable reduction following inhibition of ectonucleotidase activity suggesting that this adenosine could be derived from extracellular ATP. Interestingly, the concentrations I have measured using similar methods in the mouse preparation were elevated compared to the corresponding values obtained in the rat hippocampus. The relatively high adenosine measured in the mouse may represent a difference in signaling by extracellular adenosine

between these rodent species. It is also likely that part of the difference between our measurements and prior estimates is due to a time dependent change in biosensor sensitivity which is not accounted for in the calibration. In this study I employed paired biosensors to differentially measure adenosine while estimating inosine and downstream purine derivatives to which the INO biosensor responds. I observed a consistent change in relative sensitivity of the ADO and INO biosensors over the recording period and, based on the relative change, I estimate that our measurements are elevated by approximately 60%. Because the procedure is identical under all conditions, however, this differential sensitivity is unlikely to account for the effects of wakefulness or the disruption of wakefulness-dependent changes by dnSNARE transgene expression in astrocytes.

One intriguing aspect of the findings detailed in the third chapter is the observation that adenosine tone appears to rise rapidly within the first four hours of wakefulness and to remain relatively stable over the entire dark period. In addition, sleep deprivation maintained, but did not increase, the level of extracellular adenosine measured following spontaneous wakefulness. These observations support a model in which tonic extracellular adenosine does not gradually accumulate during wakefulness in the hippocampus but instead maintains a bi-stable equilibrium concentration with increased levels during wakefulness that rapidly decline during NREM sleep. This switch could be caused by a variety of factors including a change in the active pool of vesicles within the astrocyte, a change in adenosine metabolism or a stable modulation of astrocytic calcium signaling. In the last case, one could speculate that the apparent bi-stability could be due to a feed-forward/feed-back enhancement of net calcium signaling

by astrocytes in which ATP released from these cells could drive calcium signaling in adjacent astrocytes leading to a stable increase in regional gliotransmission. Regardless of the mechanism, however, it is clear that wakefulness increases the level of astrocyte derived adenosine. Along with the recent demonstration that astrocyte-derived adenosine can increase the surface expression of NMDA receptors (Deng et al., 2011), these results also suggest that elevated adenosine during wakefulness contributes to the enhanced NMDA receptor surface expression observed during wakefulness (Vyazovskiy et al., 2008) thereby supporting experience dependent plasticity.

Previous studies have shown that the astrocytic expression of the dnSNARE transgene attenuates the negative effects of sleep deprivation on memory consolidation (Halassa et al., 2009b), and that the disruption of late-phase long-term potentiation (L-LTP) is prevented in these mice (Florian et al., 2011). The impact of disrupting gliotransmission can be mimicked by application of adorA1 receptor antagonists, suggesting that these effects are mediated by changes in extracellular adenosine. These observations appear to contradict the finding that IP_3 dependent calcium signaling produces the opposite effect on synaptic plasticity. Having now demonstrated that extracellular adenosine is derived from an astrocytic source, however, it is possible to speculate about potential mechanisms of the astrocytic modulation of memory consolidation. There are at least two possible pathways to prevent memory consolidation. First, it is known that elevations of cAMP are critical for both L-LTP and long term memory consolidation (Abel et al., 1997). Since adorA1Rs are G_i-coupled sleep deprivation might prevent memory consolidation by causing an adorA1R-dependent reduction in cAMP. Another possibility concerns the formation of lactate which is an

important neuronal energy source and which recent results have implicated as a critical component of memory consolidation (Suzuki et al., 2011). Adenosine can stimulate glycogen synthesis (Daré et al., 2007), which is negatively coupled to lactate production (Brown and Ransom, 2007), leading to reduced lactate availability over time. Interestingly, glycogen synthesis is known to increase following extended sleep deprivation (Franken et al., 2006) suggesting that lactate levels during post-deprivation sleep might be reduced through an adenosine dependent mechanism. Under conditions of reduced lactate, which is shuttled from an astrocytic source to neurons, memory consolidation and in vivo LTP are impaired (Suzuki et al., 2011). Exogenous application of lactate restores both LTP and memory (Suzuki et al., 2011). Future studies will be needed to discriminate between these two intriguing possibilities.

Section 6.3: Allostatic Reduction of Sleep Homeostasis as a Compensatory and Potentially Maladaptive Response

The physiological consequences of accumulated sleep loss can be sustained for prolonged periods and lead to reductions in attention and performance (Van Dongen et al., 2003; Banks and Dinges, 2007; McCoy and Strecker, 2011). Despite these dramatic effects, however, the mechanisms underlying changes in brain function following periods of restricted sleep are unknown. To determine whether sleep homeostasis was significantly modified by 3 days of sleep restriction, my colleagues and I performed an acute sleep deprivation (6 h) beginning 24 h after animals were allowed to recover from sleep restriction. The results of this probe test, which are described in the fourth chapter,

revealed dramatic alterations in the homeostatic response to acute sleep deprivation: sleep restricted animals did not demonstrate the expected increase in SWA during NREM sleep nor the compensatory increase in sleep time that normally follows sleep deprivation in control non-sleep restricted mice. During the period of sleep restriction we found the expected initial increase in SWA during NREM sleep, which declined during the 3 day sleep restriction period. Consistent with previous studies (Kim et al., 2007), these results suggest there is an allostatic response to chronic sleep restriction which impairs the homeostatic response to subsequent sleep deprivation.

Enforced wakefulness increases the levels of adenosine in many brain regions including the BF, cortex and hippocampus. Because adorA1R function is required for homeostatic responses to sleep deprivation (Bjorness and Greene, 2009; Halassa et al., 2009b), we asked whether adorA1R signaling systems were dis-regulated by sleep restriction. In agreement with the observations made in chapter 3, acute (4 h) sleep deprivation using a slowly moving treadmill enhanced adenosine tone in a manner similar to gentle handling. However, the basal adenosine tone in response to spontaneous wakefulness declined during the period of sleep restriction and did not recover following either 24 h or 2 weeks of recovery sleep. Moreover, the sleep deprivation-induced increase in adenosine tone was significantly attenuated following sleep restriction. These results show a long term change in the regulation of adenosine signaling in the brain, demonstrating a powerful allostatic response to sleep restriction.

There have been conflicting results concerning allostatic responses to sleep restriction. Our results are consistent with those reported by Kim et al. (2007) and Sportiche et al. (2010), who showed a significant attenuation in SWA following sleep

restriction. However, a similar study measured SWA in discrete cortical regions (frontal, parietal and occipital) in rats, and reported that sleep homeostasis remains intact during repeated sleep restriction (Leemburg et al., 2010). These discrepancies may be due to differences in methodology and/or analysis, such as variations in sleep restriction protocols as well as differences in the methods of EEG analysis. However, consistent with these previous studies (Leemburg et al., 2010), we found a slight increase in SWA (aka SWA leakage), though non-significant, during enforced wakefulness on the treadmill. It is important to note that this compensatory increase in SWA during wake remained constant across the 3 days of sleep restriction and thus cannot account for the progressive attenuation in NREM SWA during the 2 h sleep opportunities. We also implemented two additional measurements of sleep homeostasis—adenosine tone, which is directly correlated with SWA power (Kalinchuk et al., 2011) and response to acute sleep deprivation—to further probe the consequences of accumulated sleep loss. When these two measures are used, it is clear that under the conditions of our study mice do exhibit an allostatic suppression of the sleep homeostat in response to sleep restriction. Sleep and wakefulness are not solely governed by homeostatic mechanisms but also involve intrinsic oscillations produced by the circadian pacemaker which control activity under normal conditions. Although enforcing activity during the sleep phase can produce a shift in the circadian rhythm, leading to changes in activity and hence differences in sleep pressure, several factors suggest that this is not the cause of the changes in sleep homeostasis which we observe. First, it is important to note that the diurnal variation in wakefulness and sleep is observed to some degree during and immediately following the end of sleep restriction, suggesting that the circadian rhythm is not strongly perturbed by

this manipulation. Second, adenosine tone is not only reduced at the end of subjective daytime but is also lower following sleep deprivation compared to controls. Finally, the effect of sleep restriction on both homeostatic sleep drive and adenosine tone is preserved at the start of the subjective night following two weeks of rest. At the end of this period, the pattern of wakefulness across the circadian completely returned to normal, suggesting that the component of sleep pressure driven by adenosine tone is decoupled from the circadian rhythm.

Several sources contribute to the accumulation of adenosine in the extracellular space including direct release from nerve terminals (Wall et al., 2007; Wall and Dale, 2008) and hydrolysis of ATP released from either nerve terminals or astrocytes (Wall and Dale, 2013). Adenosine can also be released through equilibrative nucleoside transporters down its concentration gradient under conditions of increased metabolic activity. Although recent studies employing conditional astrocyte specific transgenic mice have shown that ATP released from astrocytes is the dominant source of the adenosine contributing to sleep homeostasis, (Halassa et al., 2009; Fellin et al., 2009; Schmitt et al., 2012) whether sleep restriction impairs this release pathway remains to be studied. The reduction in adenosine which we observe may instead be due to reduced conversion from ATP through 5'-ectonucleotidase or increased metabolic clearance of adenosine by adenosine deaminase or adenosine kinase. Human and mouse studies (Palchykova et al., 2010; Bachmann et al., 2012; Zielinski et al., 2012) show that alterations in either of these enzymes lead to changes in adenosine dependent sleep homeostasis. Future studies will be required to identify the mechanism underlying the reduction in extracellular

adenosine and whether this sleep restriction dependent modification is the cause of the impairment of sleep homeostasis.

Recovering alcoholics often suffer severe sleep impairments that have been reported to be an effective predictor of relapse (Brower and Perron, 2010). Interestingly, a clinical study recently reported that recovering alcoholics fail to respond to acute sleep deprivation (Armitage et al., 2012). Because of these reports and given that adenosine is a known mediator of behavioral effects of alcohol (Nagy et al., 1990; Nam et al., 2012), we asked whether sleep restriction and the attendant long-term changes in adenosine signaling and sleep homeostasis might induce alterations in behavioral responses to alcohol (Nagy et al., 1990). Chronic sleep restriction significantly reduced sensitivity to the motor-impairing effects of alcohol, an impact which was sustained for at least 4 weeks after sleep restriction.

Interestingly, a recent investigation has identified regional changes in adrenergic receptor expression in response to sleep restriction and suggested that this change may be involved in allostatic sleep adaptation (Kim et al., 2013). Although our findings suggest a different mechanism for the impact of long-term sleep loss the two pathways are not mutually exclusive. Indeed, adrenergic receptors are expressed to a high degree in astrocytes (Ding et al., 2013) and recent studies have demonstrated that activation of these receptors can drive calcium signaling in astrocytes suggesting that the effect of sleep restriction on adenosine may occur through reduced activation of astrocytic release. Further studies are needed to understand the relationship between these two mechanisms and to determine whether this sleep restriction-dependent modification of adenosine signaling by astrocytes is the major cause of its impairment of sleep homeostasis.

Our results showing a sustained reduction in adenosine tone and sensitivity to alcohol following chronic sleep restriction are especially interesting in light of evidence pointing to adenosine as a known mediator of the effects of alcohol (Dohrman et al., 1997; Ruby et al., 2010; Nam et al., 2012). Ethanol increases adenosine by blocking the equilibrative nucleoside transporter ENT1 (Nagy et al., 1990), and mice lacking ENT1 have reduced adenosine tone and reduced sensitivity to alcohol (Choi et al., 2004). Ethanol-induced changes in adenosine levels can subsequently act on either A1 or A2A receptors (Dar, 1990, 2001; Micioni Di Bonaventura et al., 2012). Given the bidirectional co-morbidity between sleep disorders and chronic alcohol use, our findings pose the intriguing possibility that the adenosine ADORA1R pathway that drives sleep pressure acts on a parallel pathway which contributes to the intoxicating effects of alcohol.

One potential concern with the interpretation of the adenosine tone data presented is that the changes in adenosine we measure occur in the hippocampus, a structure not generally considered to play a role in the motor impairing effects of alcohol. In previous studies (Schmitt et al., 2012), however, we have observed that similar changes in adenosine occur in both the hippocampus and cortex in response to acute sleep deprivation, suggesting that wakefulness-dependent mechanisms controlling tonic adenosine are broadly similar in multiple brain regions. This hypothesis is further supported by the observation that blocking A1 receptors globally is sufficient to replicate the effects of chronic sleep restriction on the motor sensitivity to alcohol. Together these observations suggest a model in which perturbation of sleep homeostasis causes subsequent impairments in sleep pressure by decreasing tonic adenosine which ultimately

leads to decreased sensitivity to alcohol and subsequent changes in consumption behaviors.

An interesting parallel exists between the timecourse of reduced sleep homeostasis during chronic sleep restriction and the loss of efficacy of sleep deprivation as a therapy for major depressive disorder. Sleep deprivation provides a potent means to alleviate the symptoms of major depression in dangerously suicidal patients (Hemmeter et al. 2010) and recent results have demonstrated that one mechanism of the effect of this treatment is through elevation of adenosine (Hines et al. 2012). This therapeutic intervention has limited long term benefit, however, because it ceases to provide relief from depressive symptoms over the time course of days patients are continuously deprived of sleep. One possible explanation for this loss of effectiveness is that reduced adenosine produced as an adaptive response to compensate for the negative side effects of sleep loss prevents the therapeutic action of this molecule for depression. Further investigation would be required to determine whether this is, in fact, the case and this would potentially inform the development of treatments for major depression which target the astrocyte.

Section 6.4: Relevance of Compartment Specific Calcium-Dependent Adenosine Release for Memory Function and Consolidation

Despite the explosion of research into the function of astrocytic calcium signaling since its discovery, several aspects of this cellular response remain incompletely understood. In chapter five of this dissertation, my colleagues and I investigated the

impact of stimulating gliotransmission on synapse function within the hippocampus. To accomplish this, we have used agonists of endogenous, astrocyte enriched receptors in combination with targeted molecular genetic inactivation of IP_3 dependent calcium signaling. We found that stimulating astrocytes via the Gq coupled PAR1 receptor led to an increase in astrocyte derived adenosine and that this increase required both IP_3 signaling and vesicular fusion. Activating this pathway led to two distinct effects on synaptic transmission, both mediated by adorA1 receptor. At high levels of activation, adenosine signaling reduced excitatory synaptic transmission by inhibiting synaptic transmission. In contrast, less intense activation of PAR1 dependent calcium signaling led to an enhanced NMDA receptor dependent field response. This increase in NMDA receptor sensitivity reduced the threshold for synaptic plasticity, leading to enhanced potentiation by weak theta-burst stimulation. These results reveal that astrocytes are capable of regulating synaptic transmission in the hippocampus through calcium dependent gliotransmission and suggest that their response is graded depending on the level at which these cells are stimulated. These findings have important implications for understanding the calcium-dependent response mechanisms by which astrocytes can regulate plasticity in response to synaptic transmission.

One factor complicating the study of the underlying mechanisms controlling calcium responses within astrocytes is the high degree of nonlinearity in calcium responses within astrocytes (Rusakov et al. 2013). The InsP3Rs which mediate IP_3 dependent calcium mobilization in response following PLC activation are also sensitive to calcium elevation and may therefore engage in calcium induced calcium release, producing a feed forward increase in cytosolic calcium following small localized

elevations (Bernardinelli et al., 2011; Tanaka et al., 2013). These may be caused by IP_3 dependent signaling but might alternatively be due to the opening of calcium permeable channels or to sodium gradient reversal which can be caused by a high degree of co-transported sodium during glutamate uptake (Danbolt, 2001). Under conditions of high excitatory synaptic activity this can lead to inward transport of calcium through the sodium-calcium exchanger (NCX) potentially leading to larger calcium events (Kirischuk et al. 1997).

This high degree of compartmentalization is evident in astrocytes expressing surface targeted genetically encoded calcium indicators (GECIs) (Shigetomi et al., 2013a) in which calcium "microdomains" occur in multiple compartments of the astrocyte, particularly within the astrocytic branchlets associated with synapses (Bernardinelli et al., 2011). The close structural apposition of astrocytic processes with the synaptic cleft combined with the unusually large and complex membrane of the ER in these glial cells allows local bursts of calcium to be occur adjacent to the plasma membrane even though this calcium is liberated from intracellular stores (Pivneva et al., 2008). Although spontaneous local elevations can sum to produce larger regional calcium signals within individual astrocytes, in many studies these localized calcium events have not been observed to propagate beyond a single cell (Di Castro et al., 2011; Shigetomi et al., 2013a). This stands in contrast to astrocytic activity in cultures where calcium signals can propagate to adjacent astrocytes through direct diffusion of calcium or IP_3 through the connexin/pannexin based astrocytic "syncytium" (Giaume and McCarthy, 1996). These calcium "waves" can also jump between astrocytes via activation of P_{2y} receptors in adjacent cells by ATP released into the extracellular space (Guthrie et al., 1999;

Bowser and Khakh, 2007). Although they have been observed *in vivo* (Kuga et al., 2011) large scale calcium events of this type appear to have variable impact in intact preparations so it remains unclear to what extend they are functionally relevant (Rusakov et al., 2011). In addition, coupling between astrocytic processes is less dense and shows regional variation in the CNS (Nagy and Rash, 2003) suggesting that calcium wave dynamics may not translate in a simple manner from the equivalent version in cultured astrocytes (Rusakov et al., 2011).

In contrast to the sparse spontaneous calcium activity frequently observed *in situ*, bath application of Gq coupled receptor agonists, such as the PAR1 agonist TFLLR used in the fifth chapter of this dissertation leads to a large coordinated activation astrocyte populations similar to those produced by network stimulation such as activating sensory pathways (Takata et al., 2011). Interestingly some studies have suggested that, even when bath applied, GPCR agonists can sometimes produce compartment specific calcium signals which then propagate throughout the rest of the cell (Fiacco et al., 2007). Although our methodology did not allow us to determine if such focal events preceded the large scale, saturating calcium waves produced by TFLLR, we cannot exclude the possibility that such events take place. Moreover, our observation that different levels of astrocytic stimulation using this agonist could produce distinct, adenosine mediated effects on pre- and post-synaptic transmission suggests the possibility the response was in some way localized.

There are several potential explanations for the differential, compartment specific signaling by astrocyte-derived adenosine in response to TFLLR. Although adorA1 receptors are present at high levels in both the pre- and post-synaptic terminals (Rebola et

al., 2003), recent findings suggest that they couple to different signaling pathways depending in part on their subcellular localization. In the presynaptic terminal and soma adorA1 receptors are known to couple through the Gi-alpha subunit to reduce production of cyclic AMP. This pathway is responsible for reduced sensitivity of voltage gated calcium channels leading to inhibition by this receptor in the presynaptic terminal (Strecker et al., 2000; Arrigoni et al., 2001). Although this pathway is also active in the postsynaptic terminal, and is potentially involved in the disruptive effect of adenosine on LTP following sleep deprivation, recent studies have identified a distinct, src/fyn dependent pathway through which adorA1 receptors in the postsynaptic terminal may act to phosphorylate NMDA receptors leading greater surface stability of these receptors (Deng et al., 2011). It is possible that this pathway is more tightly coupled to adorA1 receptors allowing it to be activated by lower levels of adenosine and thereby explaining the lower observed threshold of the post-synaptic response.

Given the high degree of specialization of astrocytic filaments adjacent to synapses (Matyash and Kettenmann, 2010; Zorec et al., 2012) and the observation that calcium signaling within these cells is frequently highly localized (Bernardinelli et al., 2011), an alternative explanation for these observations is that astrocytic calcium signaling and release are compartment specific. This specificity could be due either to preferential release adjacent to postsynaptic receptors or, alternatively, to targeting of PAR1 to sites adjacent to post-synaptic targeted release sites. Because of the unique intracellular structure of the ER in astrocytes (Pivneva et al., 2008; Parpura et al., 2011), such region specific targeting can strongly affect downstream signal activation, an effect which some have suggested is responsible for the lack of impact by engineered MrgA1

receptors in astrocytes (Rusakov et al., 2011). The high degree of nonlinearity in the adenosine release provides additional support for this possibility, suggesting that a threshold activation of the PAR1 receptor leads to more global activation of astrocytic calcium signaling and hence higher release of adenosine. This is supported by the observation that multiple small elevations in cytosolic calcium can produce global calcium mobilization both in astrocytes (Bernardinelli et al., 2011) and in other cell types (Berridge, 2006). Further research will be necessary to discriminate between these possible mechanisms, and to understand the nature of the dual regulation of synaptic plasticity by astrocyte-derived adenosine.

Although astrocyte dependent changes in extracellular adenosine have been implicated in the effect of wakefulness on both behavior and synaptic plasticity (Halassa et al., 2009b; Florian et al., 2011) the timescale over which the concentration of adenosine changes in response to activation of Gq coupled receptors is smaller than the duration of wakefulness considered necessary to produce changes in global tonic adenosine (Kalinchuk et al., 2011). Moreover, the observation that adenosine can both support and inhibit hippocampal plasticity suggests that the impact of astrocyte-derived adenosine depends on how long it has been elevated. One possible explanation for this difference is that the same mechanism which enhances plasticity over the short term, increased NMDA receptor sensitivity, may produce deficits if it remains active too long. Consistent with this concept, recent studies have suggested that acute sleep deprivation is associated with net potentiation of synapses within hippocampus and that this potentiation may be involved in the detrimental effects of sleep loss. This potentiation is characterized by an increase in ionotropic glutamatergic receptor expression including

higher surface expression of AMPA as well as NMDA receptors (Vyazovskiy et al., 2008). While this increase in excitatory synaptic transmission is likely a necessary side effect of activity within the hippocampus during behaviors involving this structure, some models suggest that this form of net potentiation could prevent stabilization of new memories. In this model, increased glutamate response eventually leads to saturation of synaptic plasticity thereby reducing the signal to noise ratio and preventing efficient acquisition and storage of additional information.

By potentiating low threshold plasticity while simultaneously suppressing glutamate release, adenosine signaling may partially compensate for the saturation of excitatory neurotransmission produced by wakefulness. This would provide a homeostatic response to allow plasticity in some synapses while suppressing others, which are not currently active, through heterosynaptic depression. Based on our findings and on previous results from Deng et al. (Deng et al., 2011) this effect is likely due to increased NMDA receptor surface stability through src/fyn dependent phosphorylation coupled to postsynaptic AdorA1 receptor activation. Interestingly, however, some studies have suggested that increased NMDA receptor surface expression may be involved in the detrimental effects of sleep deprivation (Kopp et al., 2006; Longordo et al., 2009). This suggests that, at extremes, the effect of astrocyte derived adenosine on NMDA receptors may cease to be beneficial and begin to interfere with normal synaptic plasticity. In addition, extensive evidence (Florian et al., 2011) suggests that adenosine can act through Gi coupled pathways in the postsynaptic terminal to disrupt long term potentiation by inhibiting production of cyclic AMP. These mechanisms are not mutually exclusive and both may be involved in the detrimental effect of astrocyte

derived adenosine on memory consolidation. The observation that astrocyte derived adenosine can also enhance hippocampal plasticity, however, suggests that the involvement of these cells in memory function may be more complex than previously thought.

A surprising result presented in this dissertation is that extended sleep deprivation leads to allostatic suppression of the wakefulness-dependent elevation of astrocyte derived adenosine. This effect is quite long lasting, leading to reduced adenosine weeks after sleep restriction ceases. Our results clearly indicate that this reduction has several behavioral consequences including lower sleep pressure and reduced sensitivity to alcohol. Long term sleep loss is associated with a variety of deficits including problems with attention, short term memory and behavioral regulation (Banks and Dinges, 2007). It is possible that some of these deficits may also be at least partially due to the reduction of astrocyte derived adenosine that we observe. In particular, given the observation that low-threshold plasticity is enhanced by adorA1 receptor activation, it is tempting to speculate that problems with short term "working" memory under conditions of chronic sleep loss may involve a loss of astrocytic adenosine signaling. Further research will be needed to investigate these possibilities.

Section 6.5: Overall Conclusions

The findings detailed in this dissertation provide novel insight into the effect global states of waking and sleep on neuronal and astrocytic function. These behavioral states profoundly impact synaptic transmission leading to changes in behavior and in memory

function. My results indicate that they do this, in part, by controlling the interaction between neurons and astrocytes at the level of the synapse. I have identified several key effects by which wakefulness influences the level of synaptic inhibition and the threshold for NMDA receptor dependent long term potentiation by controlling the level of astrocyte derived adenosine. Specifically, I have demonstrated that: **1) Short term wakefulness increase the level of astrocyte-derived extracellular adenosine within the hippocampus through a mechanism that depends on SNARE mediated vesicular release. 2) Long term sleep disruption inactivates the effect of wakefulness on astrocyte-derived adenosine leading to a suppression of the homeostatic sleep drive and reduced sensitivity to the hypnotic effects of alcohol. 3) Calcium dependent vesicular release from astrocytes can provide a source of either adenosine or its metabolic precursors the elevation in extracellular adenosine produced by triggering this mechanism leads to distinct changes in presynaptic inhibition and NMDAR mediated LTP.** These results improve our understanding of the role of astrocytes in the CNS and also suggest a mechanism by which both short term and long term sleep deprivation can affect synaptic transmission and, ultimately, influence memory. The story is far from complete, however, and considerable work remains to understand the impact of astrocyte-derived adenosine under normal and pathological conditions.

Section 6.6: Future Directions

The observation that astrocyte-derived adenosine levels increase in response to wakefulness raises several questions regarding the mechanistic basis of this response. Because activation of GPCR dependent calcium signaling can trigger a rise in adenosine (Fig. 5.3), it is possible that this elevation is due to increased calcium dependent release of metabolic precursors of adenosine such as ATP. For this to be the case, it would require that wakefulness-history affects the basal rate of release either by increasing spontaneous calcium activity or decreasing the threshold for calcium-mediated changes in astrocyte-derived adenosine. To test these possibilities, one approach would be to measure spontaneous and induced calcium activity in astrocytes as a function of prior wakefulness. This could be done in hippocampus using calcium sensitive dyes in slices taken following various periods of spontaneous or enforced wakefulness. A more technically demanding but potentially more informative approach would be to observe calcium activity using two-photon based imaging *in vivo* using GECIs or calcium sensitive fluorescent indicators in head fixed mice implanted with a chronic cranial window (Holtmaat et al., 2011). Once the mouse was acclimated to the imaging setup (Andermann et al., 2010) calcium activity could be measured during various vigilance states after which the rate of spontaneous calcium elevations could be compared between the beginning and end of each bout of wakefulness. Alternatively, calcium activity could be measured in a similar manner using a miniaturized implantable imaging system (Ziv et al., 2013). In order to establish a causal relationship between wakefulness and changes in calcium activity the mouse could then be sleep deprived to test whether calcium activity was affected by this manipulation. Finally, the VIPP mouse model could be used to test

whether the effect of wakefulness on astrocytic calcium signaling would depend on IP3 signaling in these cells.

To determine whether the threshold for changes in astrocyte-derived adenosine is controlled by wakefulness history, one relatively simple approach would be to apply TFLLR or other means to activate astrocytic calcium, perhaps via uncaging intracellular calcium within the astrocyte (Navarrete et al., 2012), and measure the increase in extracellular adenosine following various periods of wakefulness. This could be done using adenosine biosensors in a manner similar to that described in chapter 5 (Fig. 5.3). Since passive flux of adenosine across the membrane can provide both a source (Wall and Dale, 2013) and clearance mechanism (Etherington et al., 2009), equilibrative transporters would need to be blocked to exclude the possibility that the membrane gradient can differentially influence the concentration of adenosine in response to wakefulness. An additional benefit of this approach would be that the change in adenosine produced by blocking transporters would also provide an estimate of the level of adenosine provided or removed through the passive release pathway (Pascual et al., 2005; Martín et al., 2007; Etherington et al., 2009; Ben Achour and Pascual, 2012; Wall and Dale, 2013). Based on my observations, there appears to be a diurnal variation in adenosine clearance which requires ADK activity (Fig. 3.5). Interestingly, however, this variation cannot mediate the wakefulness dependent increase in extracellular adenosine since the clearance is highest at the beginning of the dark phase which corresponds to the peak of astrocyte-derived adenosine. Further studies will therefore be needed to understand the functional significance of this change in clearance. Once the astrocyte-dependent mechanism by which wakefulness controls adenosine is better understood,

additional studies could provide details on the pathway through which wakefulness modulates astrocytic function.

In contrast to earlier studies which demonstrated that astrocyte-derived adenosine produced deficits in hippocampal plasticity, the results presented above (chapter 5) suggest that adenosine can also *enhance* synaptic plasticity. This observation suggests that astrocyte derived adenosine may play a more complex role in regulating memory function in the hippocampus than had been previously suspected. It will therefore be important in subsequent studies to untangle these effects in order to better understand the role of this signaling pathway in memory function. The mechanism we describe here only impacts low threshold plasticity. Because adenosine also inhibits synaptic transmission, thereby improving the synaptic gain of the system (Abbott, 1997) this effect may be important for incorporating new information in complex environments or after previous learning has occurred. This suggests the possibility that astrocytic calcium dependent adenosine signaling may play a role in working memory function, particularly under conditions of high memory load. One way to test this would be to determine whether inactivating IP_3 dependent calcium signaling produces deficits in spatial reversal learning. This form of training is generally conducted using either the Morris water maze or Barnes maze and requires that the animal learn a new location of a platform or escape tunnel after having already learned a different location. The presence of competing memories in this task would require one previously active representation to be suppressed in favor of a new memory. Because adenosine release in response to IP_3 dependent calcium signaling can both suppress synaptic transmission and enhance synaptic plasticity, it is well positioned to play a role in this form of learning. Since VIPP

suppresses this pathway, a potentially interesting hypothesis would be that reversal learning would be disrupted in this mouse model.

The role of astrocyte derived adenosine in short term spatial memory is another potentially worthwhile avenue for investigation. Working memory function can be disrupted by long term sleep loss (Banks and Dinges, 2007) so research aimed at understanding the role of astrocyte derived adenosine in this aspect of hippocampal function could prove quite valuable. Specifically, it would be interesting to determine whether sleep restriction, which appears to reduce adenosine mediated inhibition, would have any effect on working memory assessed via spontaneous alternation or a similar task. Understanding the precise role of astrocytes, and the adenosine signaling which they regulate, in these and other cognitive processes will shed new light on the mechanisms underlying the function of the brain and, ultimately, may lead to new means to treat neurological and psychiatric disorders.

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