REGULATION OF ADENOSINE TRANSPORTER AND AMPA RECEPTOR SUBUNIT LOCALIZATION BY PROTEIN KINASE CK2 IN RAT HIPPOCAMPUS

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ABSTRACT

The control of extracellular adenosine is crucial to the regulation of synaptic transmission and neuroprotection. Equilibrative nucleoside transporters (ENTs) are highly expressed in the hippocampus and widely accepted as critical regulators of adenosine tone. However, the mechanisms regulating the surface distribution and transport function of ENTs are largely unknown. Since ENT1 and ENT2 contain consensus sequences for phosphorylation by protein kinase CK2, and because this protein has been reported to regulate synaptic plasticity and ENT function in non-neuronal systems, the present thesis outlines the hypothesis that CK2-induced phosphorylation of ENTs is important for their cellular localization and thus the regulation of adenosine tone and synaptic transmission. Here, a functional interaction between adenosine CK2, ENTs and AMPA receptors in the hippocampus is reported. Western blot analysis shows that a variety of CK2 inhibitors (DMAT, TBB and DRB) significantly reduced the density of ENT1 and ENT2 proteins in hippocampal membrane fractions, suggesting that CK2-mediated phosphorylation of ENTs promotes their surface localization. In contrast, it was found that the ENT1 inhibitor NBTI significantly increased in the membrane localization of ENT1, relative to the control. Moreover, ENTs were found to immunoprecipitate with GluR1 and GluR2-containing AMPA receptors; and CK2 inhibitors caused a decrease in the membrane localization of GluR2 and GluR1 AMPA receptors. These results suggest a novel signaling complex linking CK2-regulated adenosine transport to AMPA receptor trafficking in the rat hippocampus. Although the physiological significance of these findings requires further investigation, this thesis provides insight into an adenosine regulation pathway that may be important for the regulation of synaptic transmission and neuroprotection in the rat hippocampus.

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotropic factor
Ca^{2+}	calcium
cAMP	cyclic-AMP
cDNA	complimentary deoxyribonucleic acid
CK1	protein kinase CK1
CK2	protein kinase CK2
CNS	central nervous system
CNT	concentrative nucleoside transporter
CPA	N^6-cyclopentyl-adenosine
CRE	cAMP response element
CREB	cAMP response element-binding
DMAT	dimethylaminotetrabromobenzimidazole
DMSO	dimethyl sulfoxide
DPY	dipyridamole
DRB	5,6-dichlororibofuranosylbenzimidazole
Ei	equilibrative insensitive nucleoside transporter
ENT	equilibrative nucleoside transporter
Es	equilibrative sensitive nucleoside transporter
GABA	γ-amino-butyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GluR	glutamate receptor
GTP	guanosine triphosphate
JNK	c-Jun N-terminal kinase

LTP	long-term potentiation
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NBMPR	nitrobenzylmercaptopurine ribose
NBTI	nitrobenzylthioinosine
NMDA	*N*-methyl-D-aspartic acid
NO	nitric oxide
PKA	protein kinase A
PKC	protein kinase C
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SAH	S-adenosylhomocysteine
T3	triiodothyronine
TBB	tetrabromobenzotriazole

CHAPTER 1

INTRODUCTION

1.1 Adenosine

Adenosine serves many important roles in the central nervous system, and it is evident that one of its major roles is as a dynamic modulator of neurotransmission. Due to the inherent neuroprotective properties of adenosine, there is an increasing amount of interest in the study of adenosine and its implications in the protection against neuronal damage that occurs during stroke and ischemic attack; however, the regulatory mechanisms for controlling the extracellular adenosine that activates its receptors requires further investigation**.**

1.1.1 Adenosinergic signaling in the nervous system

Adenosine is a well-known purine nucleoside that has many physiological implications in the central nervous system. Adenosine, as a nucleoside, plays a crucial role as a metabolic precursor in nucleic acid synthesis; in addition to its function in the control of sleep, and the coupling of cerebral blood flow to energy demand (Phillis, 1989; Brundege and Dunwiddie, 1997), adenosine has an important role as a neuroprotective agent against ischemic and seizure induced neuronal cell damage (Dunwiddie and Masino, 2001).

Adenosine acts via four subtypes of membrane receptors: A1, A2a, A2b and A3, which are coupled to G proteins. The A1 and A3 receptors are coupled to G_i proteins and cause inhibition of adenylyl cyclase, whereas the A2a and A2b receptors couple to G_s proteins that induce the stimulation of adenylyl cyclase and cause a subsequent increase in cyclic-AMP (cAMP) levels (Fredholm et al., 2005). Among the four types of adenosine receptors, A1 and A2a have the highest affinity for adenosine (~70 and 150nM, respectively), and basal extracellular levels of adenosine (~25-250nM) are enough to tonically activate a considerable proportion of these receptors (Dunwiddie and Masino, 2001; Blank et al., 2007), implying that some effects of adenosine are already exerted under basal conditions.

The A1 receptor, which has the highest abundance in the brain, has an overall inhibitory effect on neurotransmission (Dunwiddie, 1985). The inhibitory action of A1 receptors results from combined post-synaptic hyperpolarization, due to the activation of potassium channels, and the pre-synaptic inhibition of neurotransmitter release, predominantly thought to be due to inhibition of N-type calcium channels (Sebastiao et al., 2000; Cunha, 2001). In contrast, the A2a receptor, which is abundant in only a few areas of the brain, is primarily stimulatory, promoting the release of neurotransmitters pre-synaptically (Dunwiddie and Masino, 2001). Under the regulation of adenosine is the release of a wide variety of neurotransmitters, including glutamate, gamma-aminobutyric acid (GABA), acetylcholine and dopamine (Blank et al., 2007). While adenosine exerts some regulatory effect on inhibitory systems, its main effect is the suppression of excitatory glutamatergic signaling (Dunwiddie, 1985).

1.1.2 Adenosine formation

There are two well-known pathways for adenosine formation. As illustrated in Figure 1.1, adenosine can be formed either extra- or intracellularly in the CNS. Extracellularly, adenosine is produced by the metabolism of released nucleotides, by the enzyme ecto-5'-nucleotidase, which can be inhibited by ATP, ADP, and α,β-methylene adenosine diphosphate (Latini and Pedata, 2001). In addition to the formation of adenosine from nucleotides, extracellular adenosine may also be produced from the metabolism of cAMP (Latini and Pedata, 2001).

Intracellularly, adenosine is formed from the dephosphorylation of AMP by cytosolic 5'-nucleotidase; adenosine then exits the cell via bi-directional nucleoside transporters. Adenosine can also be formed intracellularly from S-adenosylhomocysteine (SAH) by SAH hydrolase, however this pathway appears to be a less significant source (Parkinson et al., 2005). These intracellular enzymes respond to only large increases in AMP during increased metabolic activity, and for this reason the intracellular formation of adenosine from catabolism of cytosolic ATP has been proposed to represent a very sensitive signal of increased metabolic rate or metabolic stress (Latini and Pedata, 2001).

Therefore, under hypoxic or ischemic conditions when intracellular ATP is depleted, intracellular formation of adenosine predominates. In contrast, under normoxic conditions, extracellular adenosine is derived mainly from released nucleotides (Latini and Pedata, 2001;

Parkinson et al., 2005). Recently, it has been suggested that astrocytes and neurons respond differently in terms of their adenosine formation when exposed to ischemic-like conditions (Parkinson et al., 2005). Under such conditions, neurons respond by producing adenosine intracellularly whereas astrocytes release adenine nucleotides that are then metabolized to adenosine (Parkinson et al., 2005).

1.1.3 Adenosine metabolism

The inactivation of extracellular adenosine is accomplished by the uptake across the cell membrane of neurons and neighboring cells, followed by either the phosphorylation of adenosine back to AMP by adenosine kinase or its deamination to inosine by adenosine deaminase (Latini and Pedata, 2001). These processes are summarized in Figure 1.1. Under normal physiological conditions, it is proposed that the predominant pathway of adenosine metabolism is phosphorylation by adenosine kinase, and that deamination becomes more important only at higher adenosine concentrations, such as under ischemic conditions. Adenosine kinase inhibitors appear to be much more effective at increasing the concentration of extracellular adenosine than adenosine deaminase inhibitors (Sciotti and Van Wylen, 1993; Pazzagli et al., 1995). It has also been shown that adenosine kinase activity is inhibited during hypoxic conditions and during the elevation of adenosine concentration (Pak et al., 1994), and that this can significantly effect synaptic transmission. This suggests an amplification mechanism for intracellular adenosine formation during metabolically stressful events, which illustrates the importance of adenosine's neuroprotective role in the CNS (Parkinson et al., 2005), as discussed subsequently.

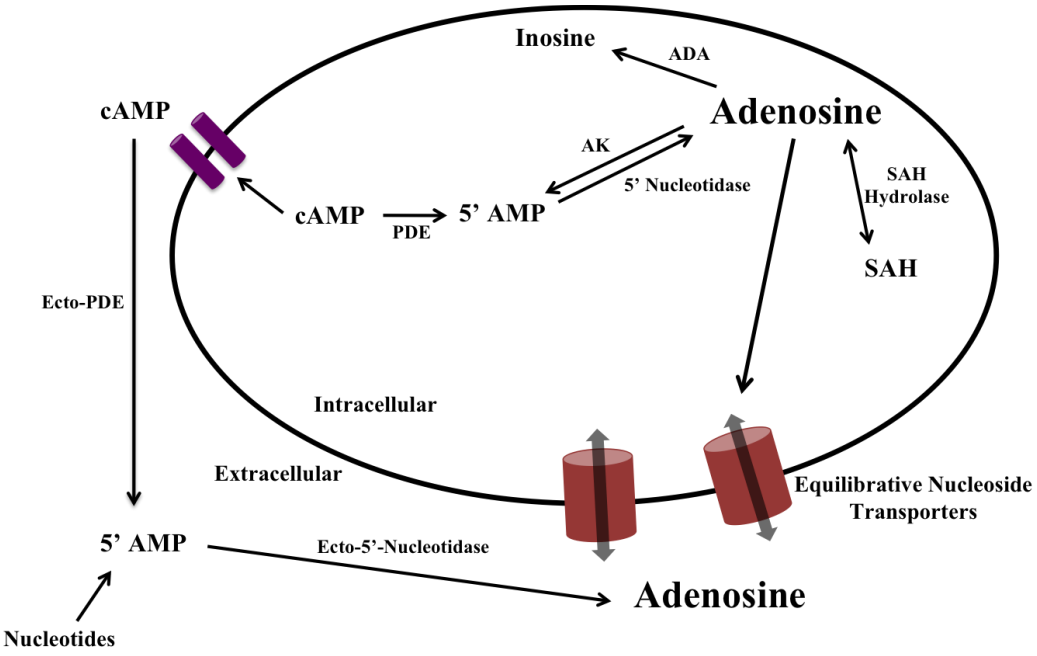

Figure 1.1. Pathways of adenosine formation and metabolism. Abbreviations are as follows: ADA, adenosine deaminase; AK, adenosine kinase; cAMP, cyclic AMP; PDE, cAMP phosphodiesterase; SAH, S-adenosyl homocysteine. Released cAMP (via non-specific, energy-dependent cAMP transporter) contributes to extracellular source of adenosine. Adapted from Latini et al., 2001.

1.1.4 Neuroprotective role of adenosine

One of adenosine's main roles in the central nervous system is as a modulator of neurotransmission. It is well known that during hypoxia, hypoglycemia or ischemia, there is an imbalance between the rates of energy demand and energy delivery; as a result, the levels of extracellular adenosine increase considerably (Cunha, 2001; Fredholm et al., 2005). This elevation in extracellular adenosine can be attributed to the release of adenosine by ischemic brain cells and from metabolized ATP derived from astrocytes (Cunha, 2001; Benarroch, 2008).

It is widely known that extracellular adenosine acts as a neuromodulator, in part, by inhibiting excitatory synaptic transmission through the activation of adenosine A1 receptors, and may also act as an endogenous neuroprotectant by reducing glutamate excitotoxicity (Dunwiddie and Masino, 2001). In models of ischemia, it has been shown that both adenosine and A1 agonists, when acutely administered, are neuroprotective. Moreover, the administration of A1 receptor antagonists can increase neuronal death in models of ischemia (Cunha, 2001; Olsson et al., 2004; Fredholm et al., 2005). However, the deletion of the A1 receptor gene in knockout

mice does not change the density of ischemic brain injury, suggesting that there are compensatory neuroprotecitve mechanisms in place that do not rely on adenosine A1 receptor signaling (Olsson et al., 2004). However, further elucidation of these neuroprotective systems in these transgenic animals is warranted.

The adenosine A2a receptor has also been shown to have implications in neuroprotection (Ongini et al., 1997; Chen et al., 1999). However, unlike the A1 receptor, activation of the A2a receptors may contribute to neuronal damage, as a massive release of excitatory amino acids during ischemia is believed to be important in subsequent neuronal death (Latini and Pedata, 2001; Nishizaki, 2004; Fredholm et al., 2005). Furthermore, A2a agonists have been shown to enhance glutamate release under ischemic as well as non-ischemic conditions, thus A2a antagonism may potentially offer neuroprotection, which has been confirmed in a global ischemia model as well as other ischemic and excitotoxicity models (Fredholm et al., 2005). Conversely, A2a receptor agonists have also been shown to elicit neuroprotective effects, however these effects are most likely mediated via the activation of non-neuronal A2a receptors and the initiation of processes such as vasodilation and the inhibition of inflammation by attenuating the release of pro-inflammatory cytokines (Cunha, 2001; Fredholm et al., 2005).

To date, there is little information about the role of A2b receptors in neuroprotection, and the literature describing A3 adenosine receptor stimulation under ischemic conditions are conflicting, some reporting beneficial and others reporting deleterious effects (von Lubitz, 1999).

1.1.5 A1 and A2a receptor signaling

Adenosine acts as a modulator at the synapse, controlling neurotransmitter release and the responsiveness of the post-synaptic cells. Adenosine accomplishes this by activating inhibitory A1 receptors or facilitatory A2a receptors; yet, adenosine's overall neuromodulatory effects are thought to result from a balanced activation of A1 and A2a receptors (Lopes et al., 1999a).

The densities of these adenosine receptors vary between different regions of the brain. A1 receptors are found mostly in neurons but are also expressed in other cells such as microglia and astrocytes, and are concentrated at the nerve terminals in the hippocampus, cerebral cortex and cerebellum (Fredholm et al., 2005). A2a mRNA is highly enriched in the striatum and in lower amounts in the lateral septum, cerebellum, cortex, and hippocampus (Fredholm et al.,

2005). However, the densities of these receptors are not static and are known to change with age (Cunha et al., 1995; Ekonomou et al., 2000). In the hippocampus, it was found that the density of A1 receptor binding in aged rats was reduced compared to young adult rats, while density of A2a receptor binding was found to increase in the hippocampus with age (Cunha et al., 1995). These changes in adenosine receptor subtypes are thought to be due to a change in expression of the receptor within the cell rather than general neuronal degeneration (Ekonomou et al., 2000).

There is increasing evidence that adenosine A1 and A2a receptors not only have opposite effects, but also interact with each other (Dixon et al., 1997; Lopes et al., 1999b; Rebola et al., 2003). This interaction has been shown to change with age. The A2a receptor-mediated facilitation of hippocampal synaptic transmission has been shown to be larger in aged than in young adult rats, and it is thought to be due to not only increased A2a receptor density in nerve terminals, but also a modified transducing system operated by A2a receptors (Rebola et al., 2003).

In synaptosomes of the striatum, and in hippocampal and cortical nerve terminals of young adult rats, it has been shown that the activation of A2a receptors causes a desensitization of A1 receptors by decreasing the binding of A1 receptor agonists, which required the activation of PKC (Dixon et al., 1997; Lopes et al., 1999b). However, in aged rats, the activation of A2a receptors no longer caused desensitization of A1 receptors, and the role of A2a receptors had changed from mostly a modulator of A1 responses in young adult rats to a direct PKA-dependent facilitation of glutamate release in aged rats (Lopes et al., 1999b; Rebola et al., 2003). It is also of note that in aged rats when compared to younger rats, an adenosine A1 receptor agonist, administered at a supramaximal concentration, was less efficient in inhibiting whereas, A2a receptor activation was more efficient at facilitating acetylcholine release, further indicating an enhanced role of A2a-specific adenosine signaling in aged rats (Cunha, 2001). It is therefore possible that, with these changes in the balance between A1 and A2a adenosine signaling, increased extracellular adenosine may lead to greater A2a receptor activation and enhanced neurotoxicity during metabolically stressful insults to the brain.

1.2 Nucleoside transporters

Adenosine, like other nucleosides, is a hydrophilic molecule and therefore relies on particular transport proteins for efficient movement across cellular membranes. Two major families of nucleoside transporters have been identified in mammalian cells, concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) (Baldwin et al., 2004; Kong et al., 2004). Within these families exist multiple subtypes which can be separated from one another based on their substrate specificity, inhibitor sensitivities, molecular characteristics and tissue distribution, although there is some overlap between the transporter subtypes (Kong et al., 2004).

Concentrative nucleoside transporters are cation symporters that mediate the unidirectional transport of nucleosides against their concentration gradient, in contrast to equilibrative nucleoside transporters, which are Na^+-independent and mediate the bidirectional flux of nucleosides across the plasma membrane in accordance with their concentration gradient. Although there are concentrative nucleoside transporters expressed in some regions of the central nervous system (Li et al., 2001; Redzic et al., 2005), the ENT subtype of nucleoside transporters appears to dominate. ENT1 and ENT2 are the two best-characterized equilibrative nucleoside transporters, and have ubiquitous tissue distribution compared to the other ENT subtypes. In addition, these ENT isoforms are highly expressed in pyramidal neurons of the hippocampus, an area of the brain that is highly susceptible to ischemic neuronal damage (Anderson et al., 1999a; Anderson et al., 1999b). Much less is known about ENT3 and ENT4, and to date they seem to only have a minor role in purinergic signaling events (Baldwin et al., 2004; Barnes et al., 2006; Parkinson et al., 2009).

1.2.1 Equilibrative nucleoside transporters: characteristics and topology

Prior to the identification of their genes in the late 1990's, members of the equilibrative nucleoside transport family were categorized based on their sensitivity to the inhibitor nitrobenzlthioinosine (nitrobenzylmercaptopurine ribose; NBMPR) (Kiss et al., 2000; Kong et al., 2004). There were two subtypes in this family, designated *es* and *ei*, corresponding to equilibrative sensitive and equilibrative insensitive, respectively (Kiss et al., 2000; Kong et al.,

2004; Podgorska et al., 2005). The *es* subtype binds NBMPR with high affinity and is inhibited at nanomolar concentrations (Kd 1-10nM), whereas the *ei* subtype is only slightly inhibited by NBMPR at high concentrations (>10μM) (Kong et al., 2004). The *es* and *ei* transporters have since been cloned and renamed ENT1 and ENT2, respectively (Griffiths et al., 1997b; Griffiths et al., 1997a; Crawford et al., 1998). In addition to ENT1 and ENT2, the cDNAs of two other mammalian ENT members (ENT3 and ENT4) have been isolated and identified (Hyde et al., 2001). All four members of the ENT family are capable of transporting adenosine, however they vary in their affinity for this nucleoside (Young et al., 2008). Where ENTs differ most in their substrate specificity is their ability to transport other nucleosides and nucleobases. ENT1 and ENT2 have similar broad substrate specificity for purine and pyrimidine nucleosides, however ENT2 possess the ability to transport nucleobases (guanine, thymine, and uracil) (Baldwin et al., 2004). The different affinities to various nucleosides and nucleobases of these ENTs are summarized in Table 1.1.

Table 1.1 Properties of mammalian nucleoside transporters

Equilibrative nucleoside transporter (ENT) family member	Number of residues	Permeant selectivity	Tissue distribution
hENT1	465	Purine and pyrimidine nucleosides	Ubiquitous
hENT2	465	Purine and pyrimidine nucleosides and nucleobases	Ubiquitous
hENT3	475	Purine and pyrimidine nucleosides and adenine (at pH 5.5)	Ubiquitous
hENT4	530	Adenosine (at pH 5.5) and organic cations, including serotonin	Heart, liver, brain
mENT1.1	460	Purine and pyrimidine nucleosides	Ubiquitous
mENT1.2	458	Purine and pyrimidine nucleosides	Ubiquitous
mENT2	456	Purine and pyrimidine nucleosides and adenine	Ubiquitous
mENT3	475	Purine and pyrimidine nucleosides and nucleobases	Ubiquitous
mENT4	528	Adenosine and adenine (at pH 5.5), organic cations not tested	Heart, liver, brain
rENT1	457	Purine and pyrimidine nucleosides	Ubiquitous
rENT2	456	Purine and pyrimidine nucleosides and nucleobases except cytosine	Ubiquitous
rbENT2	456	Purine and pyrimidine nucleosides and hypoxanthine	Ubiquitous
rbENT2A	415	Purine and pyrimidine nucleosides and hypoxanthine	Ubiquitous

Note: h, Human; r, rat; m, mouse; rb, rabbit. Reproduced with permission from Young et al., 2008.

Human ENT1 and ENT2, localized to chromosome 6p21 and 11q13 (respectively), each contain 465 residues, are ~50% identical in their sequence, and share an 11 transmembrane domain topology with an intracellular N-terminus and an extracellular C-terminus (see Figure 1.2) (Baldwin et al., 1999; Kong et al., 2004). The 457-residue rat homologue of ENT1 is 78% identical in sequence to the human ENT1 protein, and the 460-residue mouse homologue is 79% identical (Griffiths et al., 1997b; Yao et al., 1997; Handa et al., 2001; Young et al., 2008). Human ENT1 is glycosylated at Asn48, on the N-terminus in the large extracellular loop between transmembrane domains one and two (Griffith and Jarvis, 1996). This glycosylation of hENT1 may, in part, affect the binding affinity to inhibitors such as NBMPR, but is not required for its transport activity. Two glycosylation sites (Asn47 and Asn56) are present in the large extracellular loop of hENT2; while glycosylation has not been shown to be essential for ENT transport activity, it is required for efficient targeting of the protein to the plasma membrane (Ward et al., 2003; Kong et al., 2004). hENT2 also possesses a C-terminal dileucine repeat that is associated with the surface expression of hENT2 (Kong et al., 2004). It was recently discovered, by Coe et al. 2009, that mutations within a highly conserved PWN (proline,

tryptophan, asparagine) motif near the N-terminus of hENT1 disrupt plasma membrane localization in transfected MCF-7 and COS-7 cells (MCF-7, breast cancer cell line; COS-7, monkey kidney fibroblast cell line). This group also found that C-terminal mutations led to hENT1 retention within the cell (Nivillac et al., 2009).

The third subtype in the ENT family, ENT3, exhibits ~30-33% sequence identity to ENT1 and ENT2 (Hyde et al., 2001). The presence of two dileucine motifs, distinctive of endosomal, lysosomal targeting motifs, in the long hydrophilic N-terminal region of this transporter suggests that it may be an intracellular transporter (Baldwin et al., 2004; Kong et al., 2004; Podgorska et al., 2005).

The fourth transporter belonging to the ENT family, ENT4, also transports nucleosides and exhibits a novel pH-dependent adenosine transport activity that is optimal at acidic pH (Barnes et al., 2006). This transporter has an affinity for adenosine that is intermediate between ENT2 and ENT3. The 528-residue mouse homologue is 86% identical in sequence to the 530-residue human ENT4 protein (Barnes et al., 2006). It is suggested that this transporter has ubiquitous tissue distribution, and that it participates in adenosine transport under acidic conditions, such as those associated with ischemia (Barnes et al., 2006). Human and mouse ENT3 and ENT4 have glycosylation sites in the TM1–2 loop and C-terminal tail, respectively, but glycosylation status of these transporters remains unknown (Baldwin et al., 2004).

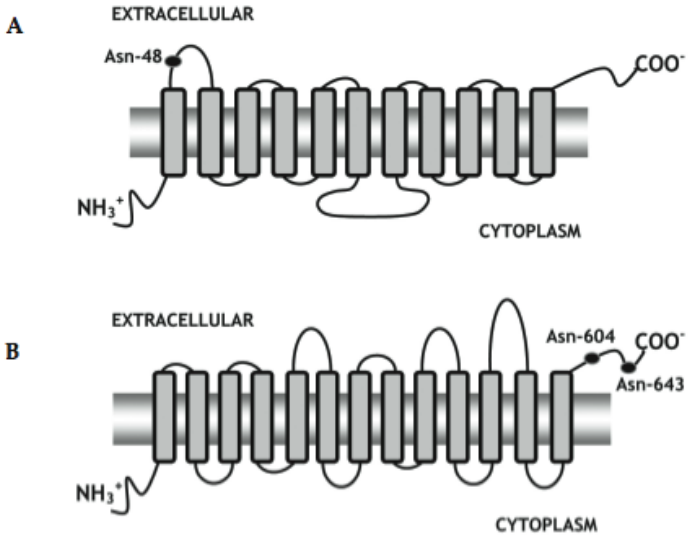

Figure 1.2. Topology of hENT1 (A) and hCNT1 (human Concentrative Nucleoside Transporter-1) (B). Grey rectangles indicate transmembrane α-helices. Black circles indicate glycosylation sites in hENT1 (Vickers et al., 1999) and hCNT1 (Loewen et al., 1999). Reproduced with permission from Podgorska et al. 2005.

1.2.2 Localization of equilibrative nucleoside transporters in the CNS

Equilibrative nucleoside transporters, in particular ENT1, but also ENT2 are considered to be ubiquitous throughout the body, although there is considerable variability in the abundance of these transporters among tissue types. ENT1 is expressed in a variety of cells, including erythrocytes and placenta, brain, liver, lung, and heart tissue (Kong et al., 2004). Likewise, ENT2 is expressed in a variety of tissues, such as vascular endothelium, heart, brain, placenta, thymus, pancreas, intestine, prostate and kidney, with a predominant expression in skeletal muscle tissue (Griffiths et al., 1997a).

In the central nervous system, the distribution of human ENT1 mRNA is widespread, with the highest levels observed in the caudate nucleus and amygdala, followed by intermediate levels in the hippocampus, subthalamic nucleus, thalamus and corpus callosum, with lower levels detected in the substantia nigra (Anderson et al., 1999b). The work of Jennings et al. in 2001 showed that the expression of hENT1 protein throughout the human brain correlated well with the distribution of hENT1 mRNA reported by Anderson et al. 1999. Human ENT1 protein was most abundant in the cerebral cortex, while moderate levels were seen in the thalamus, midbrain and basal ganglia, with the lowest amounts of hENT1 seen in the hippocampus, medulla, pons and cerebellum (Jennings et al., 2001).

RT-PCR and in situ hybridization and Northern blot revealed a wide regional distribution of rENT1 and rENT2 mRNA in rat brain, and suggested that the regional distribution of ENT1 and ENT2 is somewhat different in rat brain than in the human brain (Anderson et al., 1999a; Anderson et al., 1999b). Rat ENT1 mRNA was found in several neuron populations in the hippocampus, cortex, striatum, and cerebellum, with strong hybridization signals in pyramidal neurons of the hippocampus, granule cells of the dentate gyrus, and in cerebellar granule and Purkinje neurons (Anderson et al., 1999b). Rat ENT1 mRNA also appeared in astrocytes, vascular smooth muscle cells, and choroid epithelial cells (Anderson et al., 1999b). In another study by the same group, it was found that rENT2 mRNA is present in several brain regions, including the superior colliculus, hippocampus, cerebral cortex, striatum and cerebellum, and a variety of tissues including neurons, astrocytes, vascular smooth muscle cells and epithelial cells of the choroid plexus (Anderson et al., 1999a). However, using [^{3}H]-NBMPR autoradiography

as a method to localize ENT1 transporter proteins in the rat brain, Anderson et al. found several differences between the distribution of rENT1 mRNA and [^{3}H]-NBMPR binding sites. They found that, despite the strong in situ hybridization signal for rENT1 in the hippocampus, dentate gyrus and cerebellum, there was a relatively low density of [^{3}H]-NBMPR binding in these regions (Anderson et al., 1999b). However, these differences in the distribution of ENT1 mRNA and the transporter protein may be explained, as protein expression does not always parallel mRNA concentration. Moreover, it is possible that the ENT1 transporter protein could be located on the somatic, dendritic, or axon terminal plasma membranes, whereas the ENT1 transcript is expected to be localized in cell bodies (Anderson et al., 1999b).

A study using Western blot analysis and immunohistochemical techniques to describe the pattern of ENT1 and ENT2 transporter localization in the CNS (Alanko et al., 2006), describes findings similar to those of Anderson and colleagues. Additionally, the study describes ENT1 as being located mainly on the cellular membrane, whereas ENT2-immunoreactivity indicated cytosolic localization of the ENT2 transporter (Alanko et al., 2006). Recently, Nivillac et al. found that, in transfected MCF-7 and COS-7 cells, 70% of fluorescence tagged ENT1 that was transfected into cells was in the plasma membrane with the remainder localized intracellularly, where it is most likely undergoing processing within the secretory pathway (Nivillac et al., 2009). This evidence suggests that ENT1 may have a more important role in membrane transport of nucleosides between intracellular and extracellular regions while ENT2 may be more important for the transport of nucleosides between intracellular compartments.

Although there seems to be some species variability in the regional distribution of equilibrative transport proteins in the CNS, more than one study (Anderson et al., 1999a; Anderson et al., 1999b; Jennings et al., 2001) observed the distribution of ENT1 and ENT2 mRNA or protein in regions of the rat and human brain that are also known to contain adenosine receptors. In fact, Parkinson and co-workers showed that ENT1 and ENT2 are present in both cultured rat forebrain astrocytes and neurons; with ENT2 contributing 2-3 fold more to adenosine uptake than ENT1. These studies emphasize the importance of equilibrative nucleoside transporters in the regulation of extracellular adenosine concentration, and the correlation between ENT1 and adenosine A1 receptor distribution suggests potential interactions and/or feedback between receptors and transporters.

Overall, the broad distribution of ENTs throughout not only the CNS, but also the body as

a whole, conveys the physiological importance of these transporters in the regulation of nucleoside homeostasis.

1.2.3 Physiological relevance of equilibrative nucleoside transporters

One of the main physiological functions of nucleoside transporters is in the supply of nucleosides for salvage pathways of nucleotide synthesis in cells that lack de novo biosynthetic pathways (Baldwin et al., 2004). In addition to this, ENTs are important to the regulation of extracellular adenosine concentration, and thus they also influence the many processes that are modulated by adenosine receptor activation.

Equilibrative nucleoside transporters have been shown to have potential therapeutic application in the treatment of inflammatory and neuropathic pain, as nucleoside transport inhibitors have been reported to potentiate opioid-mediated anti-nociception in vivo (Keil and Delander, 1995). It is known that A1 receptor activation in the spinal cord has anti-nociceptive effects (Sawynok, 1998), and intrathecal administration of inhibitors of adenosine metabolism produce anti-nociception in the rat (Poon and Sawynok, 1998). There is an abundant amount of ENT1 in the dorsal horn of the spinal cord and it has been shown that inhibition by NBMPR, and a subsequent increase in adenosine concentration, leads to pre-synaptic A1 receptor-mediated inhibition of glutamatergic synaptic transmission in the substantial gelatinosa (Ackley et al., 2003). Thus, targeting nucleoside transporters has recently been recognized as a new strategy for harnessing the analgesic potential of adenosine (Yan et al., 2003).

Through the use of ENT1 knock out mice, recent studies indicate that ENT1, but not ENT2 (Nagy et al., 1990), has a physiological role in ethanol-mediated behaviors (Choi et al., 2004) and the modulation of anxiety-like behavior (Chen et al., 2007). In vitro studies show that ethanol stimulates adenosine signaling by inhibiting ENT1, whereas chronic ethanol exposure down-regulates ENT1 (Nagy et al., 1990; Mailliard and Diamond, 2004). ENT1-null mice have reduced hypnotic and ataxic responses to ethanol and greater consumption of alcohol (Choi et al., 2004). Administration of an A1 receptor agonist restored A1 receptor inhibition of synaptic transmission and reduced alcohol consumption in ENT1-null mice (Choi et al., 2004). Overall, increased alcohol consumption is linked to decreased levels of extracellular adenosine; diminished A1 receptor mediated pre-synaptic inhibition, and increased glutamate release,

leading to enhanced phosphorylation of CREB. The resultant increase in expression of CRE-regulated genes contributes to a diminished drug reward and thereby drives drug self-administration.

Nucleoside transporters also hold clinical significance as they are known to be abundant in cancerous tissue and are harnessed as a means for the cellular uptake of anticancer and antiviral drugs (King et al., 2006). Inhibitors of ENTs, dipyridamole and dilazep, are also used in the treatment of heart and vascular disease, as they have been shown to substantially increase the cardiovascular effects of adenosine (Dennis et al., 1996). Additionally, post-ischemic inhibition of nucleoside uptake by ENTs has also been shown to elevate the concentration of adenosine in the brain and prevent against ischemia-induced cell loss of hippocampal CA1 neurons in the rat (Parkinson et al., 2000). This evidence provides support that ENTs might play an important role in determining the extent of neuronal damage following a stroke.

1.2.4 Regulation of equilibrative nucleoside transporters in the CNS

Nucleoside transporters mediate the transfer of adenosine across cell membranes and therefore are largely responsible for the regulation of intracellular and extracellular adenosine. While there is an appreciable amount of data describing the regulation of other membrane transporters (Baldwin et al., 1995), little is known about the regulation of ENTs, particularly within the CNS. However, a variety of factors have been shown to influence the activity and expression of nucleoside transporters, including hormones, nutrients, adenine nucleotides, and intracellular signaling pathways (Kong et al., 2004).

1.2.4.1 ENT activity is dependent on cell cycle and growth

Within the literature, most studies looking at the regulation of ENTs suggest that ENT1 plays a role in cell proliferation, being responsible for constitutive nucleoside supply. In cultured murine bone marrow microphages, proliferation induced by M-CSF (macrophage colony-stimulating factor) resulted in the up-regulation of ENT1 mRNA and protein, but it had no effect on ENT2 expression (Soler et al., 2001). Other factors that stimulate cell proliferation and metabolism may also modulate pathways that regulate the uptake of nucleosides to accommodate

increased use of nucleosides in nucleic acid synthesis and metabolic pathways, which is supported by evidence showing that ENT1 is highly expressed in tumors (Molina-Arcas et al., 2009). Thyroid hormones are also important to the regulation of adenosine transport, as synaptosomal preparations from the brain stem of hypothyroid rats show reduced ENT1 adenosine transport, and in cultured chromaffin cells T3 (triiodothyronine) increased the number of nucleoside transporters and stimulated adenosine transport (Fideu and Miras-Portugal, 1992; Fideu et al., 1994). Additionally, it has been well documented that extracellular glucose and insulin differentially regulate the activity and expression of ENT1 and ENT2, and that the mechanisms of regulation may involve endothelial NO synthase, PKC, and mitogen activated protein kinases (MAPK) in different tissues. (Podgorska et al., 2005)

1.2.4.2 Regulation of ENTs by protein kinases

An increasing amount of literature suggests that direct post-translational modification by intracellular kinases is a possible mechanism for regulating the activity of ENTs. A study by Coe et al. 2002, using two human cancer cell lines (MCF-7 and HeLa) shows the acute stimulation of PKC causes a rapid increase in hENT1 nucleoside uptake, and that PKC isoforms δ and/or ε are responsible for this effect (Coe et al., 2002). This acute stimulation of PKC leads to an increase in hENT1-dependent nucleoside uptake that is suggested to correlate with an overall increase in the activity of the transporters at the plasma membrane, but does not appear to correlate with trafficking of proteins from intracellular stores since there was no significant difference in NBTI binding in purified plasma membranes, compared to their control (Coe et al., 2002). This is some of the first evidence that hENT1 is subject to rapid regulation by intracellular signaling pathways, however the underlying mechanisms responsible for these rapid effects are not clear but studies of other transporters suggest several possible pathways, including direct phosphorylation (Coe et al., 2002).

PKC has also been implicated in long-term regulation of ENTs, since there is a PMA-dependent (phorbol myristyl acetate) decrease in ENT1 nucleoside transport in HL-60 cells (a leukemic cell line) (Sokoloski et al., 1991; Lee, 1994). Similarly, in cultured bovine chromaffin cells, PMA treatment decreases adenosine transport (Delicado et al., 1991). It has also been shown in chromaffin cells that purinergic P2Y-receptor agonists inhibited adenosine transport,

likely through PKC activation (Sen et al., 1993). Whether the decreased transport rate by PKC activation is due to direct modification of the transporter protein or internalization of these transporters into intracellular compartments is unknown. Interestingly, a study conducted by the same group in bovine adrenal endothelial cells, showed that PKC had no effect on the ENT1 transporter in these cells (Sen et al., 1996). Coe et al showed that, in both HeLa and MCF-7 cells, long-term treatment with PMA and down-regulation of PKC is associated with a decrease in ENT1 nucleoside transport, this is in agreement with numerous other studies and suggests the possibility of transcriptional regulation (Coe et al., 2002).

In some cases, it seems as though the regulation of transporter activity by PKC is contradictive which suggests that there are many mechanisms regulating nucleoside transport function and that it is cell and tissue specific.

PKC is not the only protein kinase known to take part in the regulation of ENTs. Recently, Huang et al. showed that many protein kinase inhibitors, including those for PKC and p38 MAPK, inhibit ENT1, and possibly ENT2, transporter activity. However, Huang et al attributed this to the direct interaction with the NBMPR binding site in the transporter protein, rather than by kinase-dependent pathways (Huang et al., 2004).

1.2.4.3 CK2 modulation of ENTs

The presence of a number of kinase consensus sites within the ENT1 (and ENT2) protein, including consensus sites for PKC, PKA, CK1 and CK2, suggests the possibility of direct phosphorylation as a means of transporter regulation (Reyes et al.; Griffiths et al., 1997b). Two splice variants of mouse ENT1 (mENT1) were simultaneously identified by the Handa and Hammond laboratories (Kiss et al., 2000; Handa et al., 2001). The two variants, arising from alternate splice sites at the end of exon 7, contain differences in the region of the large intracellular loop between transmembrane (TM) regions six and seven (Handa et al., 2001). The smaller variant has a 6bp deletion in the large intracellular loop, resulting in the loss of Lys-255 and Gly-256, and conversion of the serine at position 254 to an arginine. The conversion of Ser-254 to arginine results in the negation of one of the accepted protein kinase CK2 phosphorylation consensus sites (Handa et al., 2001). Loss of this CK2 consensus site suggests that the two variants may differ in their susceptibility to phosphorylation by CK2.

In a recent study by Stolk et al., human osteosarcoma cells, which possess both ENT1 and ENT2 transport activity, transfected with catalytically active or inactive CK2α and CK2α′ subunits of CK2 were used to assess the effects of CK2 manipulation on nucleoside transporter activity. Their findings suggest that the inhibition of CK2 activity, by induction of the catalytically inactive CK2α′ subunit, lead to enhanced expression of ENT1 on the plasma membrane and functional activation of the transporter. They also found that the inhibition of CK2 activity by induction of inactive CK2α′ reduced both the activity and mRNA levels of ENT2. This suggests that ENT1 and ENT2 may be differentially modulated by CK2 and that the reduction in ENT2 activity is caused by an attenuation of ENT2 transcription, while the increase in ENT1 expression and activity is mediated by the post-translational action of CK2. Importantly, this study suggests that CK2-mediated phosphorylation of the ENTs regulates their localization on the plasma membrane and/or their trafficking between subcellular membrane compartments (Stolk et al., 2005).

In another study, looking at the modulation of mENT1 by CK2 in PK15 (nucleoside transporter deficient) transfected cells, it was found that CK2 phosphorylated ENT1 at Ser-254, and that phosphorylation at this site causes changes in the sensitivity of the transporter to the inhibitor NBMPR as well as changes in the number of functioning transporters at the plasma membrane. In this study it was found that the inhibition of CK2-mediated phosphorylation of the transporter at Ser-254 had the same functional effect as the actual loss of Ser254 in the shorter mENT1 variant, implying that Ser-254 is constitutively phosphorylated by CK2. Here, it was shown that CK2 inhibition leads to a down-regulation in the activity of ENT1 and a reduction in the number of transporters that are active at the plasma membrane. It is suggested by the authors that the reduction in the activity of ENT1, induced by treatment with the CK2 inhibitor TBB, reflects an alteration in the intracellular trafficking of this ENT1 variant such that less of the protein is recycled to the plasma membrane, as opposed to direct effect on ENT1 internalization. (Bone et al., 2007)

The opposite effect of CK2 inhibition seen in PK15 cells and the human osteosarcoma cells may reflect cell line, endogenous or recombinant ENT1, or CK2 isoform expression differences in the two experimental models (Bone et al., 2007), however, it is evident from both studies that the inhibition of CK2 influences the function and expression of ENTs.

1.2.4.4 Chronic hypoxia regulates ENT mRNA and protein levels

There are two studies that look at the effects of chronic exposure to hypoxia on ENTs (Kobayashi and Millhorn, 2001; Chaudary et al., 2004), which suggest that ENT1 gene expression may be sensitive to hypoxic regulation in both cardiac and neural tissue. The first study investigates the mechanisms of adenosine production, release, and uptake during chronic hypoxia in PC12 cells, a cell line that is used as a model of neuronal cellular physiology. In this study, the authors report that chronic hypoxia down-regulates the function of adenosine-metabolizing enzymes (adenosine kinase and adenosine deaminase), whereas it up-regulates the function of adenosine-producing enzymes (cytosolic and ecto-5'-nucleotidases) (Kobayashi and Millhorn, 2001). It was also determined that chronic hypoxia down-regulates rENT1 mRNA without significantly changing the mRNA level of rENT2. In agreement with this, Chaudary et al. 2004 determined that PKCε regulates mENT1 activity in HL-1 cardiomyocytes and a down-regulation of PKCε during chronic hypoxia results in a down-regulation of mENT1 protein and mRNA. They proposed that chronic hypoxia decreases mENT1 dependent adenosine uptake, in part, by a decrease in the amount of mENT1 protein at the membrane. In addition to a down-regulation in ENT1-mediated adenosine transport, they found evidence that suggests mENT2 is also down-regulated by hypoxia. These hypoxia-induced modifications in adenosine metabolism and transport seem to favor an increase in the extracellular concentration of adenosine, which has been shown to be neuroprotective during hypoxic insult to the brain and other tissues.

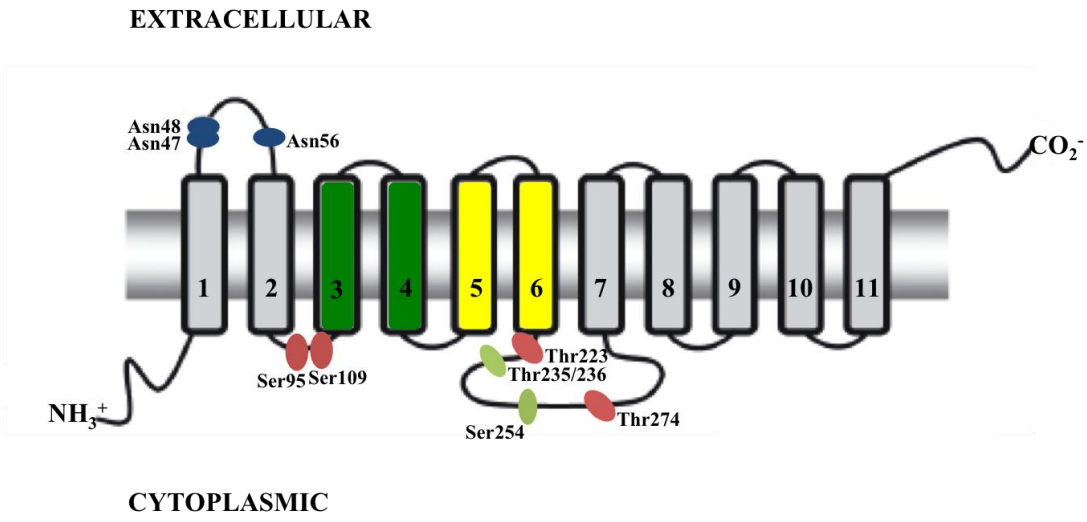

Figure 1.3. Regions of substrate interaction and points of post-translational modification in ENT1 and ENT2. Glycosylation sites are indicated by the blue circles, and correspond to Asn48 (m/hENT1), Asn47 and Asn56 (m/hENT2) (Kiss et al., 2000). CK2 phosphorylation consensus sites are depicted by green circles and correspond to Thr248 (rENT1), Ser254 (r/m/hENT1), Thr235 (rENT2), Thr236 (mENT2) and Ser270 (hENT2). PKC phosphorylation consensus sites are denoted by the red circles, which correspond to Ser109 and Thr274 (r/m/hENT1), and Ser95 and Thr223 (r/mENT2) (Data was obtained from NetPhosK and NCBI GenPept). TM regions 5-6 (yellow) are important in the recognition of nucleobases substrates (hENT1) (Baldwin et al., 2004), and TM regions 3-6 (green and yellow) are major sites of NBMPR interaction (Kong et al., 2004).

1.3 Protein kinase CK2

Protein kinase CK2, formerly known as casein kinase II, is a highly conserved, cyclic nucleotide-independent serine/ threonine protein kinase that is ubiquitously distributed in eukaryotic organisms (Blanquet, 2000; Pinna, 2002; Litchfield, 2003). CK2 is one of two casein kinases that have been found in many different cell types. There are four criteria that are used to differentiate between the two known casein kinases; these include their substrate specificity for nucleotide triphosphates, specificity for phosphate-accepting amino acids in casein, and the effects of heparin and 2,3-diphosphoglycerate on enzyme activity. The type 1 enzyme, CK1, uses only ATP as a phosphate donor, modifies serine residues, and heparin or 2,3-diphosphoglycerate does not alter its activity. In contrast, CK2 is able to use GTP or ATP as phosphate donors, modifies both serine and threonine residues, and can be inhibited by heparin and 2,3-diphosphoglycerate (Blanquet, 2000).

CK2 is a holoenzyme that is generally composed of two catalytic subunits (α and/or α') and two regulatory subunits (β) that can associate to form many distinct heterotetramers,

although free catalytic subunits can be present under some circumstances (Blanquet, 2000; Pinna, 2002; Litchfield, 2003). The exact function of the β subunit is not known, but it is suggested that it is important for stability, activity, and specificity of the enzyme (Blanquet, 2000; Litchfield, 2003), and it is suggested that the catalytic activity of the holoenzyme is higher than that of the isolated subunits (Pinna, 2002).

For a long time, it was believed that CK2 was unregulated in the cell; however there are now a number of reports that show the activity of CK2 can be regulated in neuronal and non-neuronal cells (Blanquet, 2000). In neuronal tissue, CK2 activity has been shown to change in response to treatment with brain-derived neurotropic factor (BDNF) (Blanquet, 1998), the polyamines spermine and spermidine (Tuazon and Traugh, 1991; Pinna, 2002), and during the induction of NMDA-dependent hippocampal LTP (Charriaut-Marlangue et al., 1991). It has also been shown that CK2 activity is reduced after periods of brief ischemia (Hu et al., 1993) and in response to chronic ethanol exposure (Haviryaji and Vemuri, 1997).

CK2 has a large number of substrates that possess the phosphorylation consensus sequence characterized by multiple acidic residues surrounding the phosphoacceptor residue, which can be serine, threonine or, in some cases, tyrosine (Pinna, 2002). The large numbers of known CK2 substrates include a multitude of transcription factors, many of which are nuclear proteins implicated in gene expression and transcription, as well as translational factors and a lot of other proteins with various functions in cell signaling (Pinna, 2002).

CK2 activity is present in many brain areas (cortex, septum, hippocampus, caudate-putamen, thalamus, olfactory bulb, cerebellum, and spinal cord), and immunostaining has revealed that CK2 is primarily associated with neuronal populations (Girault et al., 1990). Looking at subcellular CK2 activity, it was found that in the adult rat caudate-putamen, 45% of total CK2 activity existed in the cytosol, one-third of the activity was in the crude synaptosomal fraction, and 10% in the nuclear pellet (Girault et al., 1990). Subfractionation of the crude synaptosomal pellet further revealed that most of the CK2 activity in this fraction was associated with synaptosomes (Girault et al., 1990), indicating that CK2 is present in nerve terminals.

CK2 appears to participate in many neurophysiological functions, including neuronal differentiation, cell proliferation, and the pathogenesis of neurological diseases including schizophrenia and Alzheimer's disease (Blanquet, 2000). There is an increasing amount of evidence that suggests that CK2 participates in synaptic plasticity and memory formation. CK2

activity is transiently increased after high frequency stimulation, during the induction of hippocampal LTP (Charriaut-Marlangue et al., 1991), and BDNF, which is released in an activity-dependent manner and is important for synaptic plasticity, activates CK2 in a concentration dependent manner (Blanquet, 1998). Lieberman and Mody 1999, also demonstrated CK2 as being a selective regulator of NMDA channel gating in neurons. They showed that CK2 caused a prolongation of NMDA channel opening and that channel activity could be up-regulated by spermine through the stimulation of CK2, or down-regulated by calcineurin following activation of calcium/calmodulin (Lieberman and Mody, 1999). In addition to this, it was recently found that CK2 inhibition prevented the induction of NMDA receptor-dependent LTP, but not NMDA receptor-independent LTP (Kimura and Matsuki, 2008). It was suggested that this happens via selective regulation of synaptic NMDA receptor activity, but not extra-synaptic NMDA receptor activity, whose activation is sufficient to induce LTD. Recently, it was determined that CK2 phosphorylates NR2B, but not NR2A, subunits of the NMDA receptor, causing the endocytosis and removal of NR2B subunits from the neuronal synapse, and promoting the synaptic expression of NR2A, which is now thought to be important in the induction of LTP in young animals (Sanz-Clemente et al.).

CK2 has also been implicated as a neuroprotectant after hypoxic insult to the brain (Kim et al., 2009). It was determined that there is a reduction in CK2 activity (caused by a reduction in the catalytic subunit proteins CK2α and CK2α') after oxygen glucose deprivation and middle cerebral artery occlusion, and that loss of CK2 activity triggers ROS production via NADPH oxidase and enhanced neuronal death during ischemic injury (Kim et al., 2009).

1.4 AMPAergic glutamate receptors

Glutamate is the major excitatory neurotransmitter in the brain; its effects are mediated by the activation of ionotropic and metabotropic receptors (Santos et al., 2009). AMPA receptors, a type of ionotropic glutamate receptors, mediate fast synaptic transmission at excitatory synapses in the CNS and are crucial during neuronal development, synaptic plasticity and structural remodeling (Liu and Zukin, 2007). AMPA receptors are tetrameric assemblies of subunits GluR1-4, which are encoded by separate genes and are differentially expressed throughout the CNS (Liu and Zukin, 2007). The presence of GluR2 in heteromeric AMPA receptors governs

the channel impermeable to Ca^{2+} (Liu and Zukin 2007). Under physiological conditions, neurons of the hippocampus express GluR2-containing, Ca^{2+}-impermeable AMPA receptors (Liu and Zukin, 2007). Because these cells do not express high levels of Ca^{2+}-binding proteins or fast local Ca^{2+}-extrusion pumps, an acute loss of GluR2 would be expected to contribute to enhanced pathogenicity of endogenous glutamate and vulnerability to neuronal insults (Liu and Zukin, 2007). Ischemic insults are thought to cause remodeling of AMPA receptor subunits and a long-lasting switch in AMPA receptor phenotype, from GluR2-containing to GluR2-lacking receptors (Liu and Zukin, 2007). Thus, global ischemia, such as that which occurs during a stroke, causes delayed neuronal death, primarily of hippocampal CA1 neurons, which has been suggested to be caused by increased Ca^{2+} entry through AMPA receptors and subsequent excitotoxicity (Gorter et al., 1997).

As mentioned previously, it is well known that there is an increase in the level of extracellular adenosine during ischemia, and preliminary findings from our research group point to a novel mechanism whereby the glutamate AMPA receptor subunits undergo redistribution during prolonged adenosine A1 receptor activation. However, the relationship between the rise in extracellular adenosine and AMPA receptor subunit remodeling is not yet well understood. Two serine/threonine kinases that are widely expressed in the brain, p38 MAPK and c-Jun N-terminal kinase (JNK), have recently been shown by Brust and colleagues to be an important link between adenosine A1 receptor activation and the inhibition of synaptic transmission pre-synaptically. It has been shown that adenosine A1 receptor activation increased the phosphorylation and activity of p38 MAPK and JNK, and that this increased activity initiates a signaling cascade that inhibits glutamate release from pre-synaptic terminals (Brust et al., 2006; Brust et al., 2007). The synaptic depression induced by receptor A1 activation was attenuated by JNK inhibition as well as p38 MAPK inhibition. The inhibition of p38 MAPK also inhibited adenosine A1 receptor activation dependent phosphorylation of JNK (Brust et al., 2007). A role for p38 MAPK and JNK in the redistribution of GluR2 receptors was suggested in a study done on the responsiveness of AMPA receptors in the nucleus accumbens after removal of repetitive cocaine treatment (Boudreau et al., 2007). It was shown that phosphorylation of JNK following cocaine challenge was inversely related to surface/intracellular GluR1 and GluR2 ratios. Also, after saline challenge, the increased phosphorylation of p38 MAPK correlated with a decrease in the surface/intracellular ratio of GluR1 and GluR2 (Boudreau et al., 2007). Therefore, based on

the evidence that suggests the involvement of p38 MAPK and JNK in both adenosine A1 signaling cascades pre-synaptically as well as in AMPA receptor subunit translocation, a possible link between adenosine A1 receptor activation and AMPA receptor trafficking may exist. Thus, the persistent activation of adenosine A1 receptors could lead to AMPA receptor subunit redistribution and a novel form of synaptic depression.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

The aim of this thesis is to examine the localization of equilibrative nucleoside transport proteins 1 and 2 (ENT1 and ENT2) in response to the inhibition of protein kinase CK2. Furthermore, the localization of specific glutamate receptor subunits (GluR1 and GluR2) will be assessed, as this may be an indicator of the importance of ENT regulation in synaptic signaling.

Specific Aims:

1. To observe changes in membrane localization of ENT1 and ENT2 induced by CK2 inhibition.

Equilibrative nucleoside transporters (ENT1 and ENT2) contain strong consensus sites for CK2 phosphorylation and it has been suggested that CK2-induced phosphorylation of ENTs may increase nucleoside transport activity and/or membrane localization (Kiss et al., 2000; Handa et al., 2001; Stolk et al., 2005; Bone et al., 2007). CK2 mediated phosphorylation of ENTs would thus provide a novel mechanism for the regulation of extracellular adenosine tone and synaptic transmission in the brain. However, it remains unclear whether this phosphorylation of ENTs by CK2 is physiologically significant. Therefore the first objective of this thesis is to observe the membrane localization of ENT1 and ENT2 in response to CK2 inhibition.

Hypothesis:

The inhibition of CK2 activity, through the prolonged incubation of hippocampal tissue slices with CK2 inhibitors DMAT, TBB and DRB, will cause a reduction in the surface localization of ENT1 and ENT2.

2. To investigate any changes in the membrane localization of AMPA receptor subunits, GluR1 and GluR2, induced by the inhibition of CK2 activity.

It has been demonstrated that ischemic events, during which there is a rise in extracellular adenosine, can cause a remodeling of AMPA receptor subunits such as to allow for greater Ca^{2+} permeability of the cell (Gorter et al., 1997; Liu and Zukin, 2007). However, the relationship between the rise in extracellular adenosine and the AMPA receptor subunit remodeling is not yet well understood. Preliminary findings from our research group points to a novel mechanism whereby the glutamate AMPA receptor subunits undergo redistribution during prolonged adenosine A1 receptor activation. If CK2-mediated phosphorylation is important for the surface localization of ENTs, inhibition CK2 activity may lead to a rise in extracellular adenosine concentration and a subsequent redistribution of AMPA receptor subunits. Therefore, the second objective of this thesis is to determine whether CK2 inhibition also alters the membrane localization of GluR1 and GluR2 AMPA receptor subunits.

Hypothesis:

Inhibition of CK2 activity leads to a reduction in the surface localization of AMPA receptor subunits GluR1 and GluR2.

3. Since it is known that the concentration of extracellular adenosine can vary with temperature, which may be attributed to the altered activity of the ENT2 adenosine transporter (Dunwiddie and Diao, 2000), the third objective of this thesis is to determine whether variation in the temperature that the experiments are conducted at makes ENT1 or ENT2 more or less susceptible to regulation by direct inhibition with ENT inhibitors.

Hypothesis:

Changing the temperature that the experiments are performed at does not affect the extent to which direct ENT inhibition changes the surface localization of ENT1 or ENT2.

CHAPTER 3

MATERIALS AND METHODS

3.1 Hippocampal slice preparation

Sprague-Dawley rats at postnatal day 18-30 (P18-30) (Charles River, Canada) were anesthetized with halothane and immediately decapitated according to protocols approved by the University Committee of Animal Care and Supply (UCACS). Brains were readily extracted and placed in ice-cold oxygenated dissection medium containing the following (in mmol/L): 87 NaCl, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 7.0 $MgCl_2$, and 0.5 $CaCl_2$. Hippocampal slices (400μm thick) were cut using a vibrating tissue slicer (Leica VT1200S) and maintained for 1 hr at either room temperature (22-24°C) or 35°C (water bath) in artificial cerebro-spinal fluid (aCSF) containing the following (in mmol/L): 126 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.0 CaCl, and aerated with 95% O_2/5% CO_2. Hippocampal slices prepared from five animals were used for each experiment day (independent experiment). Hippocampal slices were then incubated, with shaking, either at room temperature or 35°C in various treatments: DPY (10μmol/L), DMAT (5μmol/L), TBB (10μmol/L), DRB (100μmol/L), CPA (500nmol/L) or NBTI (10nmol/L or 100nmol/L) for 1.5 hr (CPA for 90min, 45min or 30min). DMSO was used as a vehicle control. The tissue was lysed and separated into membrane and cytosolic fractions, whole cell, or into synaptosomal fractions (see below).

3.2 Drugs

Dipyridamole (DPY), 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB), 5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (DRB), adenosine N^6-cyclopentyladenosine (CPA), and S-(4-Nitrobenzyl)-6-thioinosine (NBTI) were all obtained from Sigma-Aldrich and made up as stock solution before being added to aCSF. The final concentration of DMSO was always <0.1%.

NBTI and DPY are structurally distinct ENT inhibitors that bind with high affinity to sites closely adjacent to the nucleoside recognition site (Young et al., 2008). NBTI binds with

high affinity (K_i 0.1-10 nM) to ENT1, but has little effect on ENT2 at concentrations less than 1 μM (Baldwin et al., 1999). Alternately, DPY, while displaying large sensitivity differences among species, inhibits both rat ENT1 and ENT2 with similar affinity (K_i 5-30 μM) (Griffith and Jarvis, 1996).

3.3 Membrane and cytosolic fractionation

Membrane and cytosolic fractions were prepared as previously described (Brust et al., 2006; Brust et al., 2007). The hippocampal tissue slices were lysed in a solubilization buffer (30 min) that contained 50mmol/L Tris (pH8.0), 150mmol/L NaCl, 1mmol/L EDTA, 1mmol/L NaF; protease inhibitors (Sigma): 1mmol/L PMSF, 10μg/μL aprotinin, 10μg/mL pepstatin A, 10μg/mL leupeptin, 2mmol/L Na_3VO_4, 20mmol/L sodium pyrophosphate, 3mmol/L benzamidine hydrochloride, and 4mmol/L glycerol 2-phosphate. The tissue homogenates were then centrifuged at 1,000 g (5 min at 4°C) to remove cellular debris. Membrane and cytosolic fractions were separated by centrifugation at 13,000 g for 1 hr at 4°C by omitting the detergent from the solubilization buffer. Proteins from the particulate (membrane) fraction were resolved in normal solubilization buffer (as above plus detergent, 1% NP-40) after removal of cytosolic extract (supernatant).

3.4 Whole cell fractionation

For whole cell fractionation, the lysis of hippocampal tissue slices was carried out as for membrane and cytosolic fractionation except 1% NP-40 was added to initial solubilization buffer. Following tissue lysis, samples were centrifuged at 1,000 g for 5min. Proteins from the supernatant (whole cell fraction) were then resolved in the solubilization buffer containing 1% NP-40. This was achieved by vortexing the sample every 5min for 20min. Protein concentration of each sample was then determined, as described below.

3.5 Synaptosomal fractionation

Synaptosomes were prepared from the hippocampal tissue slices (mentioned above) following previously published methods (Hammond and Clanachan, 1985; Parkinson et al., 2009). Hippocampal tissue slices were homogenized in 10 volumes ice-cold 0.32M sucrose and centrifuged at 1000 g for 10 min at 4°C. The pellet was washed twice and the pooled supernatants were centrifuged for 55mins at 17,000 g (4°C) to obtain crude synaptosomal pellet. The pellet was re-suspended in HEPES buffer, containing the following (in mmol/L): 100 NaCl, 25 glucose, 68.3 sucrose, 5.3 KCl, 1.8 CaCl, 1.0 MgSO4, 20 HEPES; pH 7.4.

3.6 Western blot analysis

All protein concentrations of the samples were determined by a dye binding method (Bradford assay), using bovine serum albumin as a standard. The Bradford assay was carried out using protein assay dye from BioRad and measured using a photospectrometer (Novaspec Plus visible spectrophotometer from Biochrom). Hippocampal homogenates were diluted with 1X Laemmli sample buffer (BioRad) plus 100mmol/L dithiothreitol (DTT; Sigma) and boiled for 10 min. 30-50µg/lane proteins were separated on 10% polyacrylamide gel (180V for ~1 hr) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (0.4A for 3 hr at 4°C) (Millipore). The membranes were incubated overnight (4°C) with the appropriate primary antibody diluted in 5% non-fat milk in TBST containing 0.025% sodium azide. The primary antibody dilutions are as follows:

Table 3.1 Primary antibody descriptions

Primary Antibody	Source	Dilution	Sequence
Polyclonal rabbit anti-ENT1	Millipore	1:1000	mENT1
Polyclonal rabbit anti-ENT1	Abcam	1:1000	250-350 of hENT1
Polyclonal rabbit anti-ENT2	Abcam	1:1000	350-450 of hENT2
Polyclonal rabbit anti-GluR1	Millipore	1:1000	276-287 of rGluR1
Polyclonal rabbit anti-GluR1	Abcam	1:1000	850-C-terminus of hGluR1
Monoclonal mouse anti-GluR2	Millipore	1:1000	175-430 of mGluR2
Polyclonal rabbit anti-synapsin 1a/b	Santa Cruz	1:1000	C-terminus of hSynapsin 1a/b
Monoclonal mouse anti-GAPDH	Millipore	1:2000	Clone 6C5 rGAPDH

Note: h, human; r, rat; m. mouse.

Following four washes (15 min) with TBST, the membranes were incubated with rabbit, mouse or goat horseradish peroxidase-conjugated secondary antibody against IgG (1:1000; Santa Cruz, 1:3000 for GAPDH) in 5% non-fat milk (1 hr, room temperature). The membranes were then washed 4X (15 min) with TBST, and proteins were visualized using enhanced chemilluminescence (ECL).

3.7 Immunoprecipitation

To examine interactions of ENT1 (Abcam) and ENT2 (Abcam) with GluR1 (Abcam) and GluR2 (Millipore) AMPA receptor subunits, immunoprecipitation was performed by first incubating 500μg extract from hippocampal homogenates with 4μg of the antibody corresponding to the protein being immunoprecipitated (overnight; 4°C). Then protein A/G PLUS agarose (Santa Cruz) beads were added to the homogenates for either an additional 3 hours (at room temperature) or overnight (4°C) to collect the immunoprecipitates. Agarose beads were then collected by pulse spins, and washed four times with wash buffer (solubilization buffer containing 0.1% NP-40). Proteins from the agarose beads were eluted with 65 μl of 1X Laemmli sample buffer, boiled for 10 min, and resolved in polyacrylamide gels. Proteins were then electrotransferred to PVDF membranes and proteins were visualized using ECL.

3.8 Protein densitometry and statistics

Protein densitometry analysis of Western blots was performed using QuantityOne software from BioRad and ImageJ software from NCBI. Protein signals were quantified by normalizing all bands with the GAPDH protein band signals; protein bands for each of the treatments were then normalized to their control. Significant differences between population means were assessed using a one-way ANOVA followed by a Student-Newman-Keuls post-hoc statistical test, where appropriate, with a confidence level of $p<0.05$. Results are presented as mean ± standard error of the mean (SEM). Each Western blot, or repetition, was considered to be n; multiple n values were taken from each experiment day, or independent experiment. All statistical analyses were performed using the software program INSTAT 2.

CHAPTER 4

RESULTS

4.1 Effect of protein kinase CK2 inhibition on ENT1 and ENT2 protein localization

Little is known about how the activity and/or localization of ENTs are controlled in the hippocampal brain region. However, it has been shown that the localization of nucleoside transporters is subject to regulation by kinase-dependent pathways. Both activators and inhibitors of PKC, PKA and CK2 have been shown to alter the transport function and membrane localization of nucleoside transporters in a variety of cell lines (Lee, 1994; Coe et al., 1996; Coe et al., 2002; Stolk et al., 2005; Bone et al., 2007). Indeed, a mouse ENT1 splice variant that is lacking CK2 phosphorylation consensus sites has been shown to have a reduced concentration of functional ENT1 transporters at the level of the plasma membrane, compared to its larger variant (Handa et al., 2001; Bone et al., 2007).

So while the manipulation of PKC and CK2 activity has been shown to modify ENT1 plasma membrane localization and cellular nucleoside transport capacity (Coe et al., 2002), whether the regulation of ENTs by CK2 phosphorylation in the hippocampus is physiologically significant remains unclear. Multiple Western blot experiments, measuring the protein level of each nucleoside transporter (ENT1 and ENT2), were performed to examine the effect of protein kinase CK2 inhibition on membrane localization of ENTs.

4.1.1 ENT1 membrane localization

Firstly, the effect of the inhibition of CK2 function, by DMAT, TBB and DRB, on the surface localization of ENT1 in native hippocampal tissue slices was determined. The polyclonal antibody against ENT1 (Millipore) recognized protein bands at approximately 83 (Figure 4.1C), 60 and 50 kDa (Figure 4.1A, C). Although the ENT1 protein is most often detected around 50 kDa, it has been observed to be anywhere between 40-62 kDa, with the differences attributed to differential glyclosylation (Reyes et al.; Torres et al., 1992; Yao et al., 1997; Musa et al., 2002).

The protein band observed at approximately 83 kDa has recently been suggested by other groups to represent an ENT1 protein dimer or ENT1 protein complex (Reyes et al.; Torres et al., 1992), however it was not analyzed in this study due to the high saturation of this band in most of the blots that were obtained, making it difficult to measure the densitometry accurately. Minor bands of lower molecular mass seen in Figure 4.2A probably represent degradation products of the rENT1 polypeptide.

Densitometry analysis of the 60 kDa band revealed that CK2 inhibition with DMAT (5μmol/L), TBB (10μmol/L) and DRB (100μmol/L) caused significant reduction in the amount of protein localized at the level of the plasma membrane ($p<0.01$). Compared to the control treatments, the amount of ENT1 in the hippocampal cell membrane fraction was reduced by 43 % (0.56855 +/- 0.08351), 33 % (0.66971 +/- 0.09337) and 38 % (0.61948 +/-0.0925) by DMAT, TBB and DRB, respectively (Figure 4.1B). Analysis of the cytosolic fraction of the tissue samples revealed no significant change in the cytosolic localization of ENT1 among the 60 kDa band after treatment with CK2 inhibitors, when compared to the control (Figure 4.2B).

Analysis of the 50 kDa protein band showed that there were no significant changes in the membrane or cytosolic localization of ENT1 in the presence of the CK2 inhibitors (Figure 4.1D, Figure 4.2C).

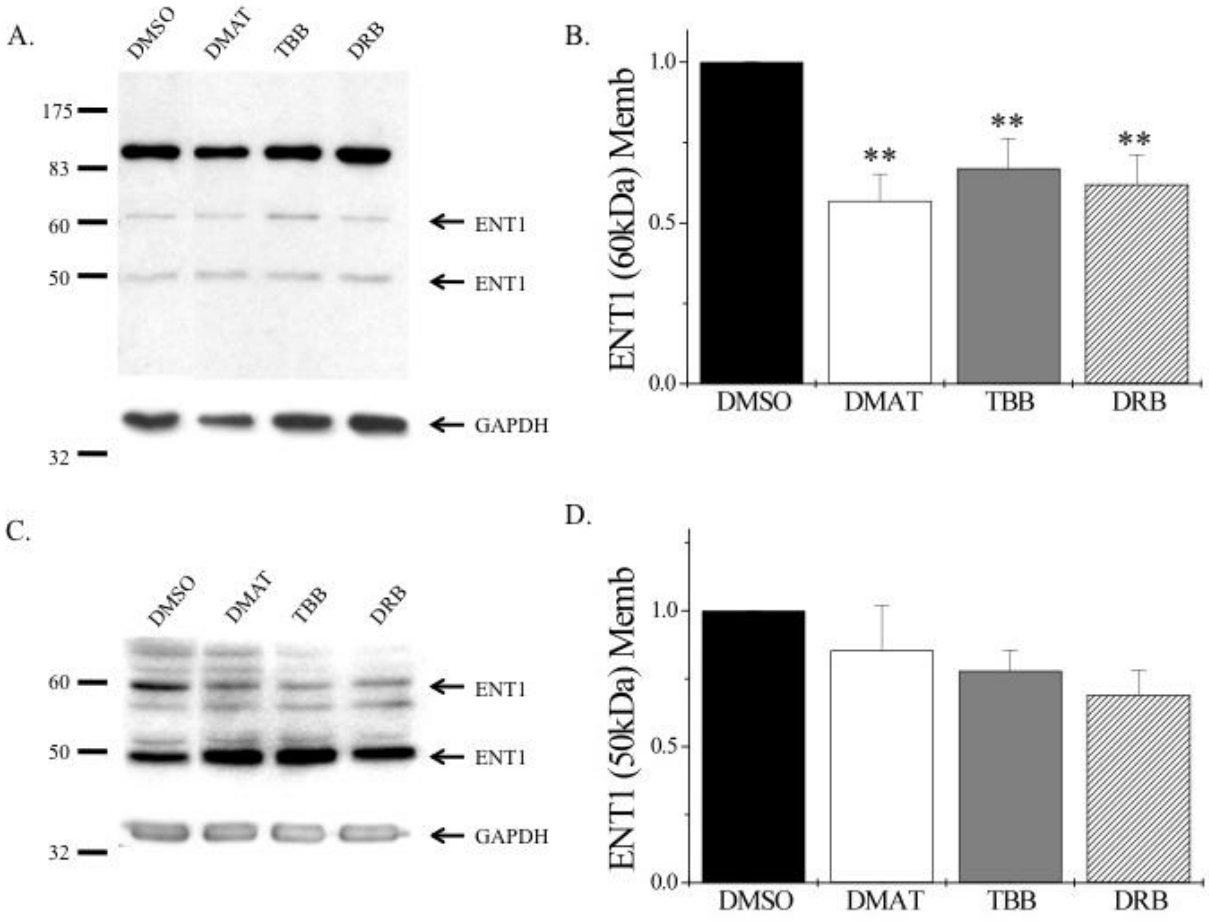

Figure 4.1. CK2 inhibition decreases ENT1 localization in membrane fractions. Hippocampal slices were exposed to either control DMSO, DMAT (5μM), TBB (10μM) or DRB (100μM) for 1.5hr. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A.** Inhibition of CK2 causes internalization of ENT1 in membrane fractions. *Top.* Full-length representative Western blot showing ENT1 bands near 83 kDa, 60 kDa and 50kDa, and the GAPDH protein bands (~37 kDa) used to normalize ENT1 signals (*Bottom*). **C.** *Top.* Partial Western blot showing ENT1 bands near 60 kDa and 50 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT1 signals. **B, D.** Quantitative representation of multiple Western blots showing inhibition of CK2 caused internalization of the 60 kDa ENT1 protein in membrane fraction ($p<0.01$, one-way ANOVA), but not the 50 kDa protein ($p=0.1936$, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=8), DMAT (n=7), TBB (n=6) and DRB (n=8); from three independent experiments] [ENT1 50 kDa- DMSO (n=10), DMAT (n=10), TBB (n=10), DRB (n=9); from three independent experiments]. **$p<0.01$ vs. control.

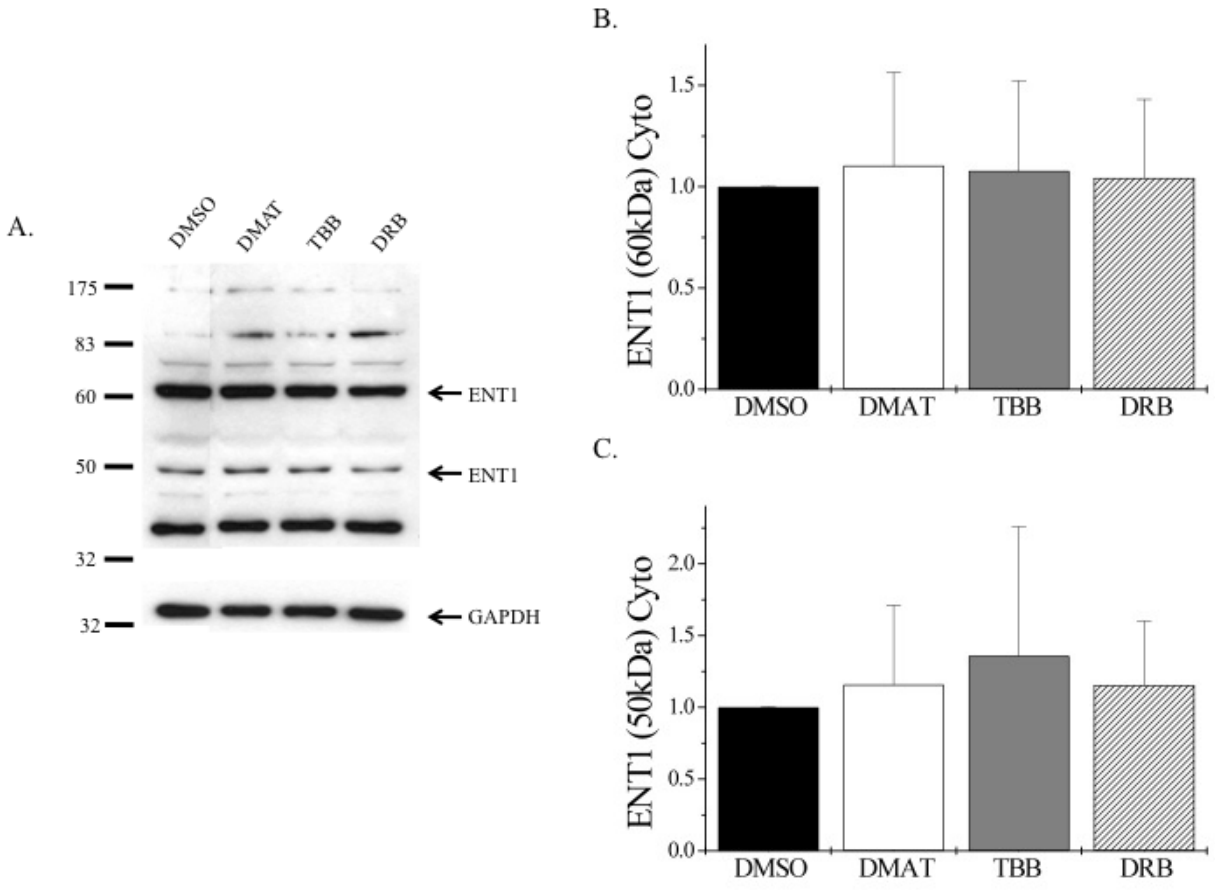

Figure 4.2. CK2 inhibition does not alter cytosolic localization of ENT1. A. Inhibition of CK2 does not cause internalization of ENT1 in cytosolic fractions. *Top.* Representative Western blot showing ENT1 bands near 50 kDa and 60 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT1 signals. **B-C.** Quantitative representation of multiple Western blots showing inhibition of CK2 did not cause a significant change in the localization of the 60 kDa or 50 kDa ENT1 proteins (p=0.9675 and p=0.9852, respectively, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=5), DMAT (n=5), TBB (n=4) and DRB (n=5); from three independent experiments] [ENT1 50 kDa- DMSO (n=5), DMAT (n=4), TBB (n=3), DRB (n=5); from three independent experiments].

4.1.2 ENT2 membrane localization

Western blot analysis of ENT2 revealed protein localization similar to that of ENT1, with a pronounced protein band at 83 kDa (not shown in figure) and two other protein bands near 60 kDa and 50 kDa, as seen in Figure 4.3A. We found significant changes among treatment groups for the 60 kDa protein ENT2 protein ($p<0.001$), but not for the 50 kDa band ($p>0.05$) (Figure 4.3 B, C) in membrane fractions. The protein band near 83 kDa was not analyzed. Comparing the treatment groups to the controls, we observed that DMAT (5μmol/L), TBB (10μmol/L) and DRB (100μmol/L) reduced the membrane localization of the 60 kDa ENT2 by 32 % (0.68507+/- 0.08686; $p<0.01$), 56 % (0.43618 +/- 0.06179; $p<0.001$) and 53 % (0.46946 +/-0.09648; $p<0.001$), respectively (Figure 4.3B). There was no significant change in the cytosolic localization of either the 50 kDa or 60 kDa protein band among the treatment groups, when compared to the control (Figure 4.4).

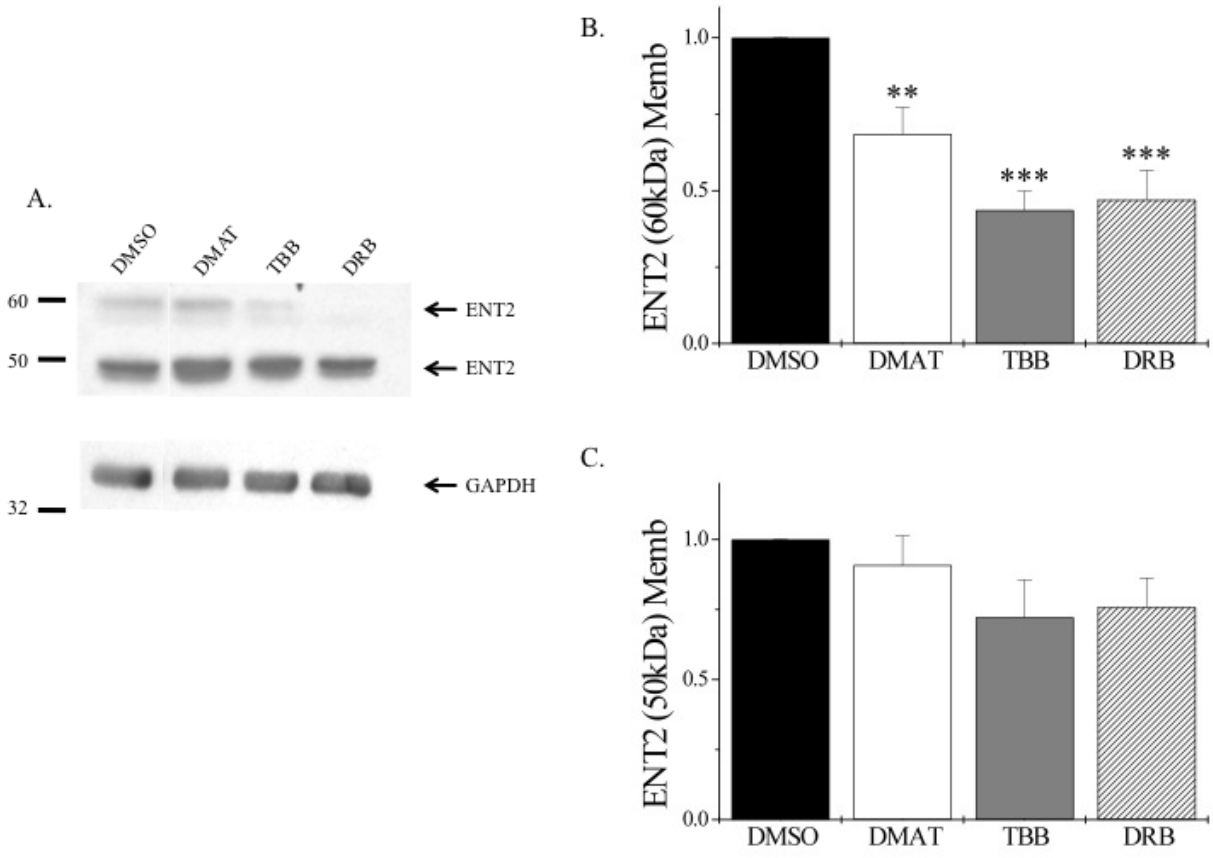

Figure 4.3. CK2 inhibition decreases ENT2 localization in membrane fractions. Hippocampal slices were exposed to either control DMSO, DMAT (5μM), TBB (10μM) or DRB (100μM) for 1.5hr. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A.** CK2 inhibition causes ENT2 to be reduced in membrane fractions. *Top.* Representative Western blots showing ENT2 bands near 50 kDa and 60 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT2 signals. **B-C.** Quantitative representation of multiple Western blots showing inhibition of CK2 caused a significant reduction in ENT2 membrane localization ($p<0.0001$, one-way ANOVA). Values for ENT2 (60 kDa) are means ± SEM from 8 independent blots from four experiments (n=8). $^{**}p<0.01$, $^{***}p<0.001$ vs. control. ENT2 (50 kDa) signals from the different treatments [n=6 for all treatments] did not differ significantly ($p=0.1957$).

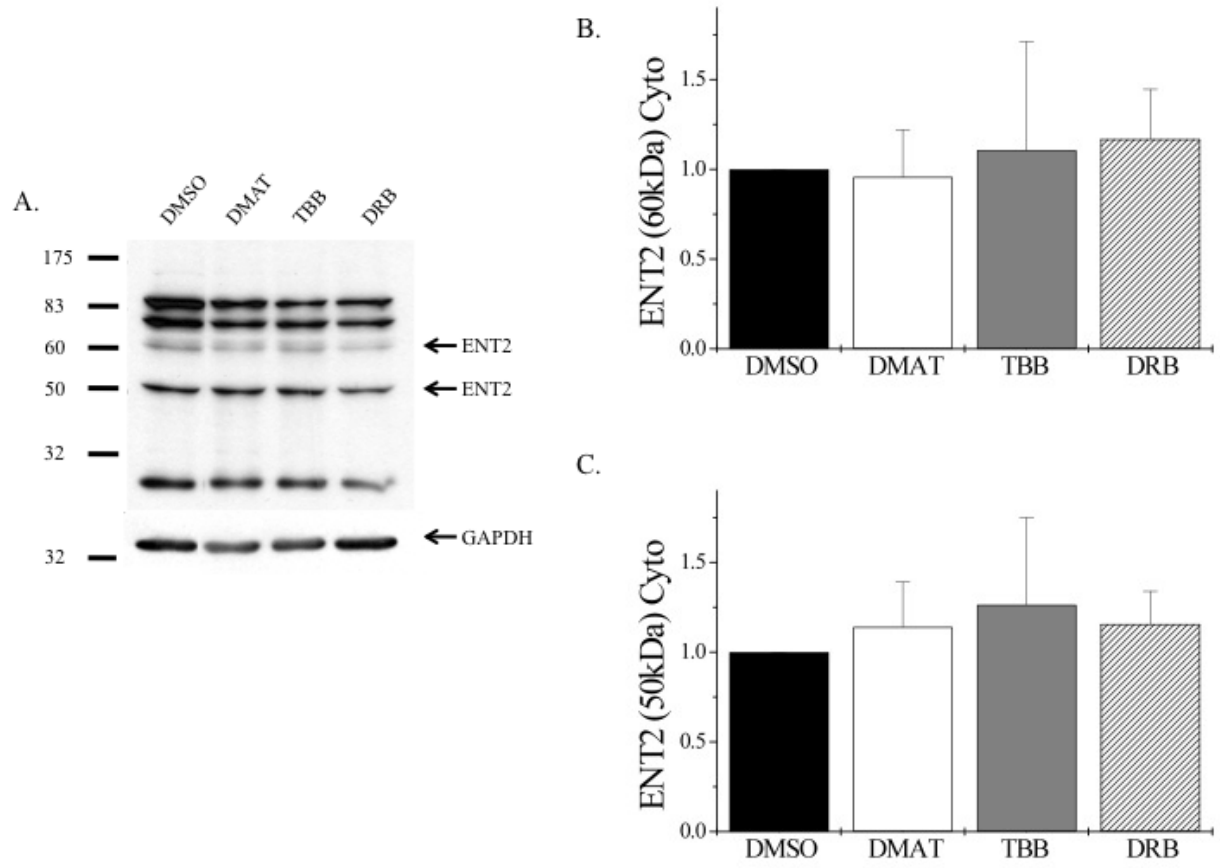

Figure 4.4. CK2 inhibition does not alter cytosolic localization of ENT2. Hippocampal slices were exposed to either control DMSO, DMAT (5μM), TBB (10μM) or DRB (100μM) for 1.5hr. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A.** Inhibition of CK2 does not cause internalization of ENT2 in cytosolic fractions. *Top.* Representative Western blots showing ENT2 bands near 50 kDa and 60 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT2 signals. **B-C.** Quantitative representation of multiple Western blots showing inhibition of CK2 does not cause significant change ($p>0.05$) in the 60 kDa ($p=0.9463$) or 50 kDa ($p=0.8655$) ENT2 protein in cytosolic fractions. Values are means ± SEM [ENT2 60 kDa- DMSO (n=3), DMAT (n=3), TBB (n=2) and DRB (3); from three independent experiments] [ENT2 50 kDa- DMSO (n=7), DMAT (n=6), TBB (n=2), DRB (n=7); from five independent experiments].

4.1.3 ENT1 and ENT2 localization at the neuronal synapse

Because ENT1 and ENT2 are localized in glial cells as well as neuronal cells, a clear disadvantage of using the hippocampal slice preparation and membrane and cytosolic fractionation was that the two cell types could not be separated. Thus, a synaptosomal preparation was used to get a clearer understanding of what was happening at the nerve terminals, relative to the rest of the tissue.

To verify our synaptosome isolation procedure, protein samples were probed with synapsin 1a/b (Santa Cruz), a pre-synaptic marker that recognizes synaptic vesicle-associated phospho-proteins. A strong protein signal was detected for synapsin at approximately 80 kDa (Figure 4.5), suggesting that our samples were enriched synaptosomes. As with the membrane and cytosolic preparations, the ENT1 and ENT2 antibodies (Abcam) recognized protein bands at 50 and 60 kDa, as shown in Figure 4.6A, C and Figure 4.7A, and at 83 kDa (not shown in figure) in the crude synaptosomal fractions. However, unlike the hippocampal membrane fraction, there was no significant change in the localization of ENT1 or ENT2 among any of the CK2 treatment groups at the neuronal synapse, for both the 50 and 60 kDa protein bands, nor was there a significant change in ENT1 or ENT2 localization at the neuronal synapse with application of 500nmol/L CPA (an adenosine analogue) for either 90 or 30 min, or 10μmol/L of the direct ENT inhibitor DPY, (Figure 4.6B,D and Figure 4.7B, C).

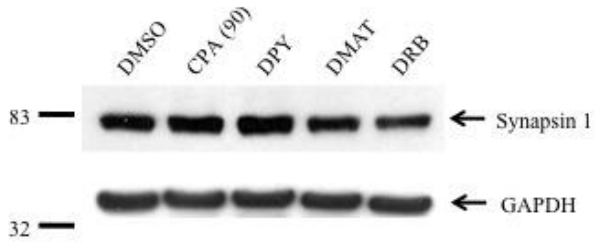

Figure 4.5. Synapsin Western blot. *Top.* Representative Western blot showing synapsin band signals near 80 kDa in the synaptosomal fraction of hippocampal slices. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize synapsin signals. CPA, adenosine N^6-cyclopentyladenosine (adenosine analogue); DPY, dipyridamole (ENT inhibitor).

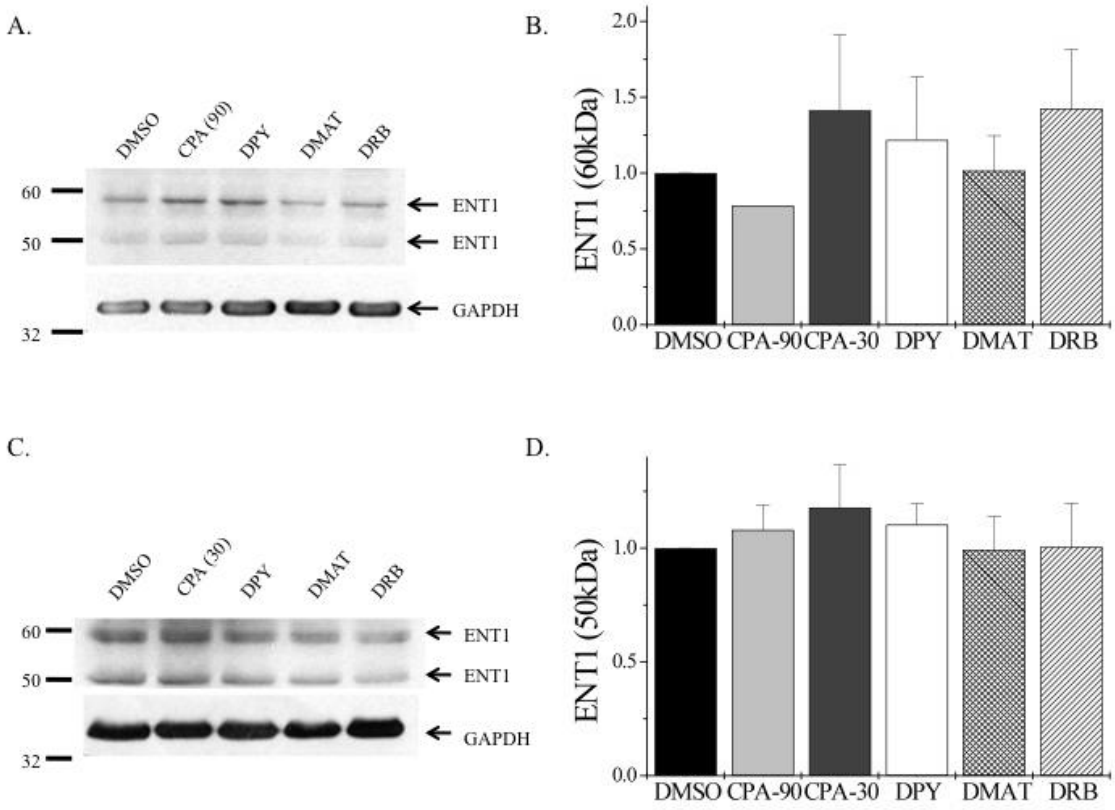

Figure 4.6. Localization of ENT1 in synaptosomal fractions following the inhibition of CK2 and ENTs. Hippocampal slices were exposed to either control DMSO, CPA (500nM), DPY (10μM), DMAT (5μM), or DRB (100μM) for 1.5hr (or 30 min for CPA). Hippocampal homogenates were separated into crude synaptosome fractions used for Western Blot analysis. **A, C.** Inhibition of CK2 or ENT, or application of CPA did not cause a significant change in ENT1 localization at the synapse. *Top.* Representative Western blots showing ENT1 bands near 60 kDa and 50 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT1 signals. **B, D.** Quantitative representation of multiple Western blots showing that CPA or inhibition of CK2 or ENT did not significantly change ENT1 localization in the synaptosomal fraction. (p=0.8388, 60 kDa ENT1; p=0.9381, 50 kDa ENT1, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=6), CPA (90 min) (n=2), CPA (30 min) (n=4), DPY (n=6), DMAT (n=6), and DRB (n=6)] [ENT1 50 kDa- DMSO (n=8), CPA (90 min) (n=4), CPA (30 min) (n=4), DPY (n=8), DMAT (n=8), and DRB (n=8)]. CPA, adenosine N^6-cyclopentyladenosine (adenosine analogue); DPY, dipyridamole (ENT inhibitor).

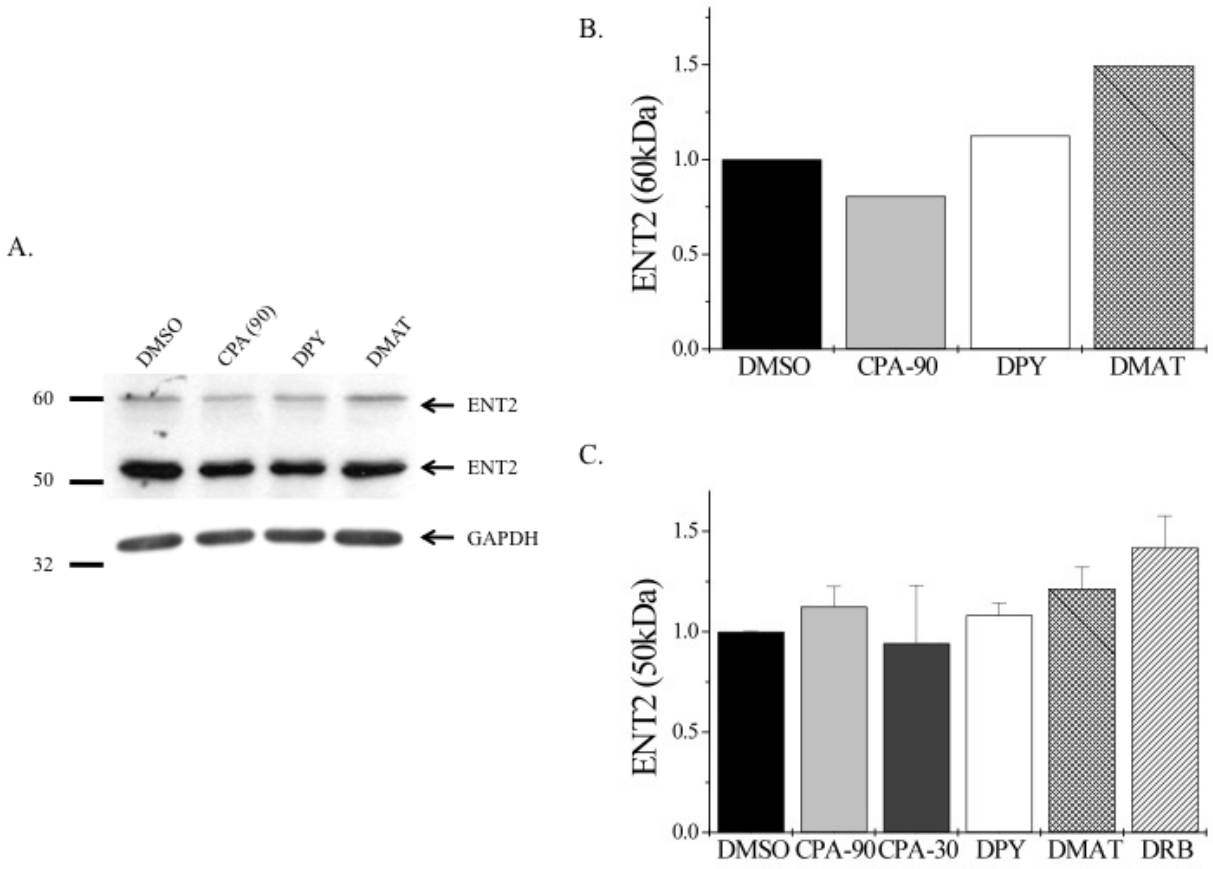

Figure 4.7. Localization of ENT2 in synaptosomal fractions following the inhibition of CK2 and ENTs. Hippocampal slices were exposed to either control DMSO, CPA (500nM), DPY (10μM), DMAT (5μM), or DRB (100μM) for 1.5hr (or 30 min for CPA). Hippocampal homogenates were separated into synaptosomal fractions and used for Western Blot analysis. **A.** Inhibition of CK2 or ENT, or the application of CPA did not cause a significant change in ENT2 localization in synaptosomal fractions. *Top.* Representative Western blots showing ENT2 bands near 60 kDa and 50 kDa. *Bottom.* GAPDH protein bands (~37 kDa) used to normalize ENT2 signals. **B-C.** Quantitative representation of multiple Western blots showing that the application of CPA or the inhibition of CK2 or ENT did not significantly change ENT2 localization in the synaptosomal fraction. (p=0.0879, 60kDa ENT2; p=0.0574, 50 kDa ENT2, one-way ANOVA). Values are means ± SEM [ENT2 60 kDa- DMSO (n=2), CPA (90 min) (n=2), DPY (n=2), and DMAT (n=2)] [ENT2 50 kDa- DMSO (n=10), CPA (90 min) (n=7), CPA (30 min) (n=3), DPY (n=10), DMAT (n=9), and DRB (n=6)].

4.2 ENTs are physically associated with proteins involved in synaptic transmission

Immunoprecipitation is the technique that is used to isolate and concentrate a known protein of interest. With co-immunoprecipitation, it is possible to identify unknown members of a larger protein complex by targeting a known protein with a specific antibody and thus isolating it as well as all other proteins that are physically associated with it. Here we used co-immunoprecipitation to identify other proteins that may be physically associated with ENT1 and ENT2 to gain more knowledge of ENT signaling in the rat hippocampus.

Previous reports show that ENT inhibition raises extracellular adenosine tone and causes synaptic depression (Dunwiddie and Masino, 2001), however it is not yet known whether changes in glutamate receptors accompany this effect of ENT inhibition. Here, immunoprecipitation experiments were performed to determine whether the GluR1 or GluR2 AMPA receptor subunits exist in the same signaling complex as ENT1 or ENT2 in the rat hippocampus. Immunoprecipitation of whole cell hippocampal lysates with rabbit anti-ENT1 or rabbit anti-ENT2 antibodies (Abcam) and subsequent immunoblotting with polyclonal rabbit anti-GluR1 (Abcam) revealed the presence of GluR1 proteins in both ENT1 and ENT2 immunoprecipitates (Figure 4.8, Top, lane 2 and 3). These protein bands were not present when the immunoprecipitating antibody was omitted (Figure 4.8, Top, lane 1). Subsequently, the immunoprecipitated ENT1 and ENT2 proteins were immunoblotted with monoclonal mouse anti-GluR2 (Millipore) and the presence of GluR2 proteins was detected in both ENT1 and ENT2 immunoprecipitates (Figure 4.8, Bottom, lane 2 and 3). Again, the protein bands were not present when the immunoprecipitating antibody was omitted (Figure 4.8, Bottom, lane 1), serving as a negative control. This data suggests that both GluR1 and GluR2 are physically associated with ENT1 and ENT2 and that there is an intimate relationship between adenosine signaling and synaptic transmission in the rat hippocampus.

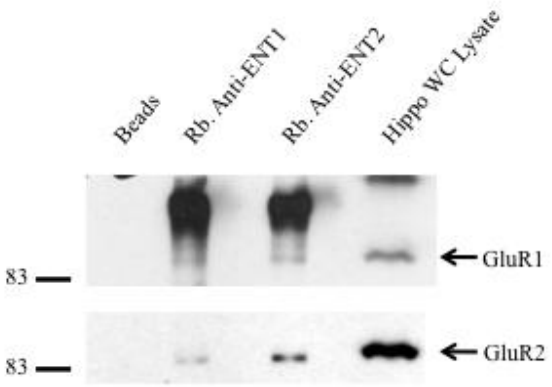

Figure 4.8. Adenosine transporters physically associate with AMPA receptor subunits. Immunoprecipitation (IP) data showing that ENT1 and ENT2 proteins are physically associated with GluR1 and GluR2 AMPA receptor subunits. *Top.* Protein A/G-PLUS- Agarose (negative control) and whole cell lysates were used in IP. Whole cell lysates immunoprecipitated with rabbit anti-ENT1 (Abcam), rabbit anti-ENT2 (Abcam), and whole cell lysates (positive control). GluR1 protein bands are recognized at ~106 kDa (Abcam) and GluR2 protein bands exist near 96 kDa (Millipore).

4.3 Inhibition of protein kinase CK2 alters AMPA receptor localization

Western blot analysis was performed to evaluate the effects of CK2 inhibition on the surface localization of the AMPA receptor subunits, GluR1 and GluR2 (Figure 4.9, 4.10). Since we have found a novel interaction between ENTs and GluR1 and GluR2-containing AMPA receptors (see above, Figure 4.8), we next tested whether the inhibition of CK2 function with DMAT, TBB and DRB also altered the membrane localization of GluR1 or GluR2 in native rat hippocampal tissue slices. We found, from five independent experiments, that inhibition of CK2 activity caused a significant reduction in the amount of GluR1 present at the plasma membrane ($p<0.0001$). Compared to control groups, DMAT (5μmol/L), TBB (10μmol/L) and DRB (100μmol/L) reduced the membrane localization of GluR1 by 29% (0.71255 +/- 0.07724; $p<0.001$), 35% (0.64942 +/- 0.07265; $p<0.001$) and 35% (0.64824 +/-0.06258; $p<0.001$), respectively (Figure 4.9B). However, it was apparent, from the densitometry of two reproducible blots showing the cytosolic expression of GluR1, that there was no change in the GluR1 cytosolic localization after CK2 inhibition (Figure 4.9C, D).

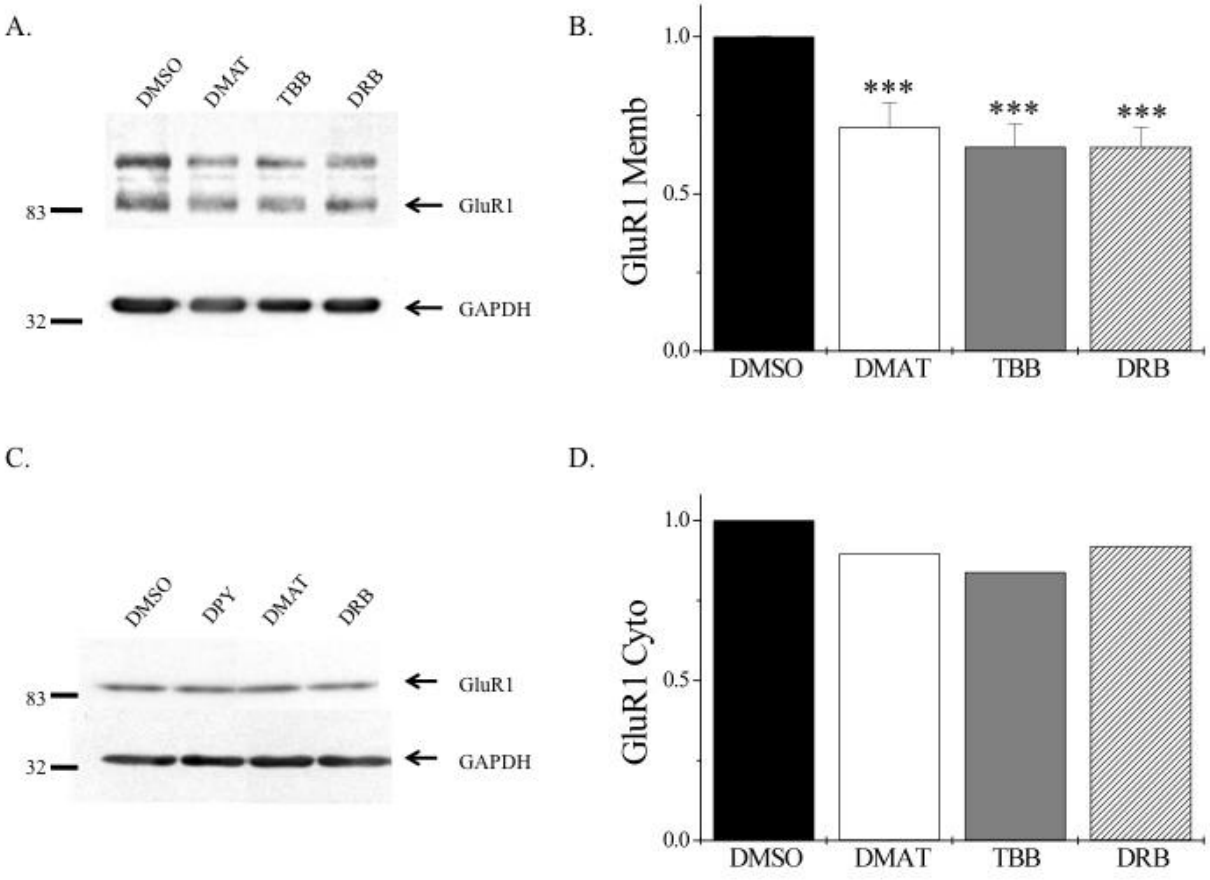

Figure 4.9. Inhibition of CK2 causes a reduction in membrane localization of GluR1. Hippocampal slices were exposed to either control DMSO, DMAT (5μM), TBB (10μM) or DRB (100μM) for 1.5hr. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A.** *Top*. Representative Western blots showing that GluR1 band signals in the membrane fraction, near 106 kDa, are decreased by CK2 inhibitors. *Bottom.* GAPDH protein bands (~37 kDa) are used to normalize GluR1 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of CK2 caused GluR1 internalization in membrane fraction ($p<0.0001$, one-way ANOVA). Values are means ± SEM [DMSO (n=12), DMAT (n=10), TBB (n=11) and DRB (n=12); from five independent experiments]. **$p<0.001$ vs. control. **C.** Representative Western blots showing that there is no change in the GluR1 band signals in the cytosolic fraction by CK2 inhibitors. **D.** Quantitative representation of two Western blots showing that there was no apparent change in the cytosolic localization of GluR1 by CK2 inhibition or direct ENT inhibition [n=2 for all treatments].

Similarly, when compared to the control groups, the amount of GluR2 present at the plasma membrane after treatment with DMAT (5μmol/L), TBB (10μmol/L) and DRB (μmol/L) was reduced by 41% (0.59286 +/- 0.13991; $p<0.01$), 56% (0.4387 +/- 0.06655; $p<0.001$) and 40% (0.60376 +/-0.06737; $p<0.01$), respectively (Figure 4.10B). Values are from four independent experiments with an overall significance of $p<0.0001$. Alternately, there was no significant change ($p>0.05$) in the cytosolic localization of GluR2, compared to the DMSO control (Figure 4.10D).

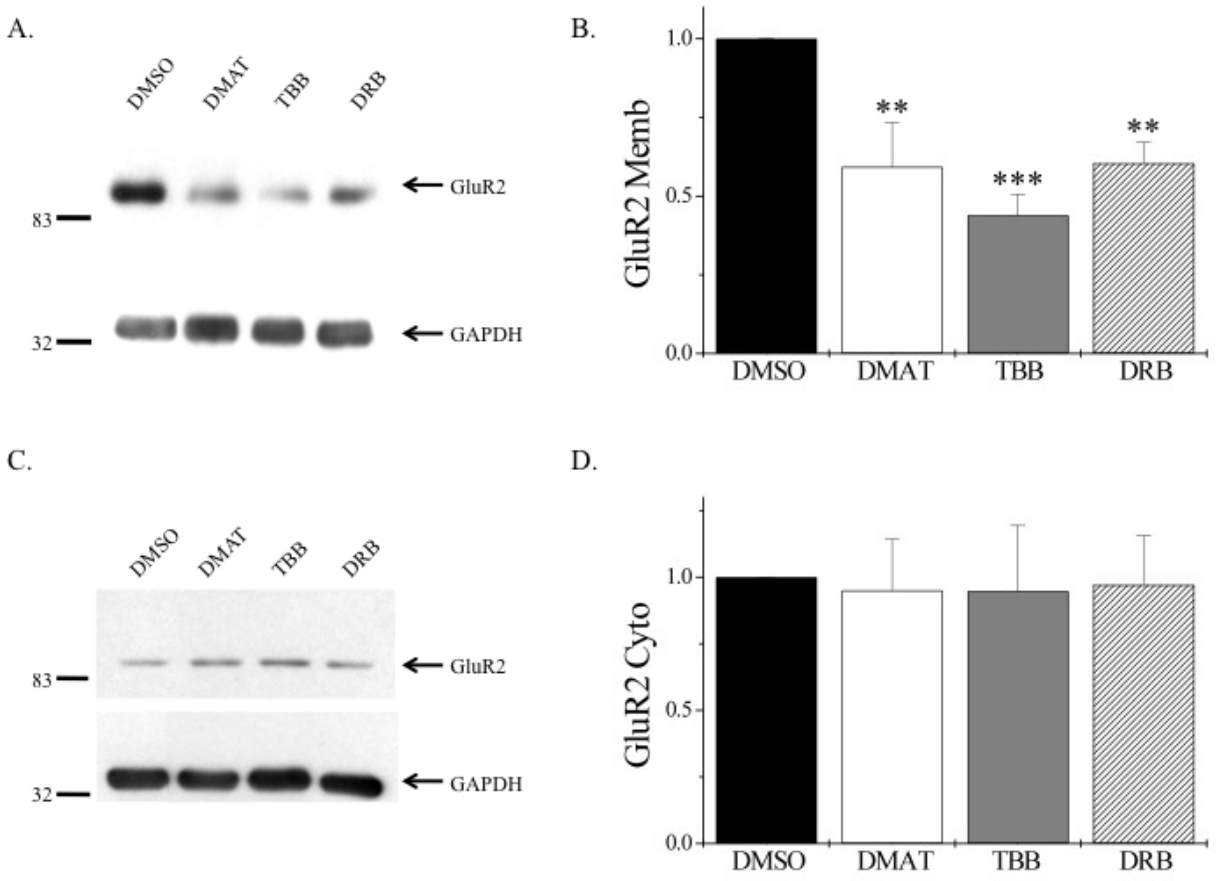

Figure 4.10. Inhibition of CK2 causes a reduction in membrane localization of GluR2 . Hippocampal slices were exposed to either control DMSO, DMAT (5μM), TBB (10μM) or DRB (100μM) for 1.5hr. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A.** *Top*. Representative Western blots showing that GluR2 band signals near 96 kDa are decreased by CK2 inhibitors. *Bottom.* GAPDH protein bands (~37 kDa) are used to normalize GluR2 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of CK2 caused a reduction of GluR2 in the membrane fraction ($p<0.0001$, one-way ANOVA). Values are means ± SEM [DMSO (n=13), DMAT (n=12), TBB (n=12) and DRB (n=12); from four independent experiments]. **$p<0.01$ and ***$p<0.001$ vs. control. **C.** *Top.* Representative Western blots showing that there is no change in the GluR2 band signals in the cytosolic fraction by CK2 inhibitors. *Bottom.* GAPDH protein bands (~37 kDa) are used to normalize GluR2 signals. **D.** Quantitative representation of multiple Western blots showing that there was no significant change ($p=0.9955$, one-way ANOVA) in the cytosolic localization of GluR2 by CK2 inhibition [DMSO (n=8), DMAT (n=6), TBB (n=5), DRB (n=8); from four independent experiments].

Together, these results suggest that GluR1 and GluR2-containing AMPA receptor trafficking from the membrane coincides with a similar pattern of ENT trafficking from the membrane after CK2 inhibition. However, similar to what we found with ENT1 and ENT2, densitometry analysis of GluR1 and GluR2 at the neuronal synapse revealed that there was no significant change ($p>0.05$) in the localization of either GluR1 or GluR2 in this region of the cell after incubation with CK2 inhibitors (DMAT and DRB for GluR2) or the direct ENT inhibitor DPY, or the adenosine analogue CPA (Figures 4.11, 4.12). This suggests that the regulation of GluR1 and GluR2 localization by CK2 inhibition may be specific to certain regions of the nerve cell.

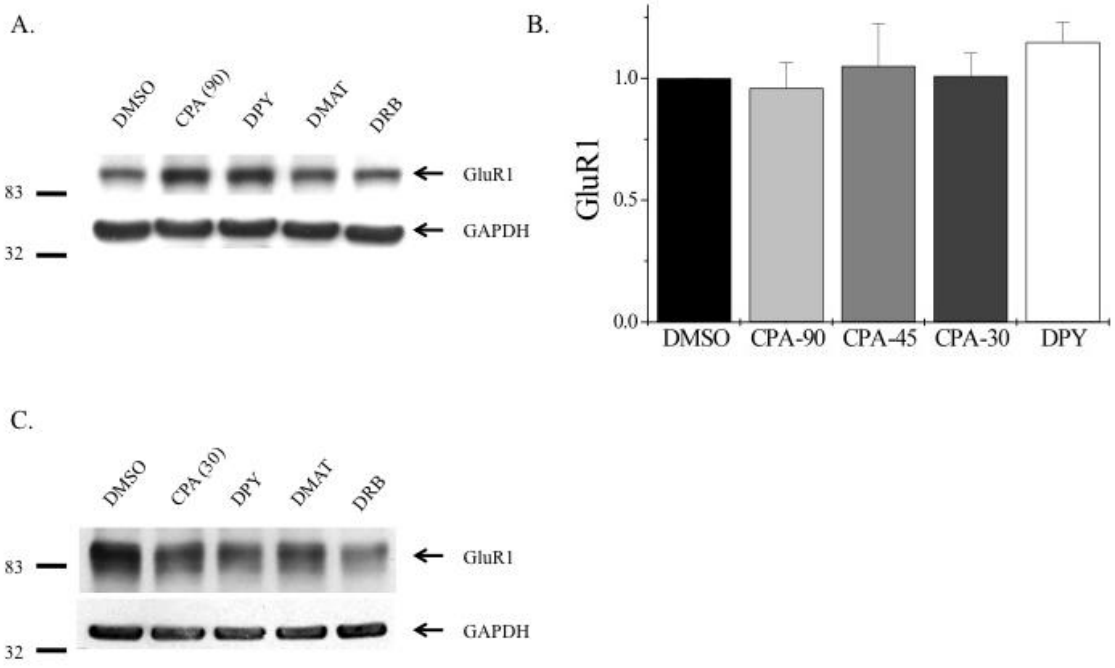

Figure 4.11. The effect of CPA and ENT inhibition on the localization of GluR1 in synaptosomal fractions. Hippocampal slices were exposed to either control DMSO, CPA (500nM; 90, 45 0r 30 min), DPY (10μM; 1.5hr). Hippocampal homogenates were separated into synaptosomal fractions and used for Western Blot analysis. **A, C.** Inhibition of ENT did not cause a significant change in GluR1 localization in synaptosomal fractions. Representative Western blots showing GluR1 bands near 106 kDa. GAPDH protein bands (~37 kDa) used to normalize GluR1 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of ENT did not significantly change GluR1 localization in the synaptosomal fraction. (p=0.7765, one-way ANOVA). Values are means ± SEM [DMSO (n=11), CPA (90 min) (n=8), CPA (45 min) (n=6), CPA (30 min) (n=10) and DPY (n=5)].

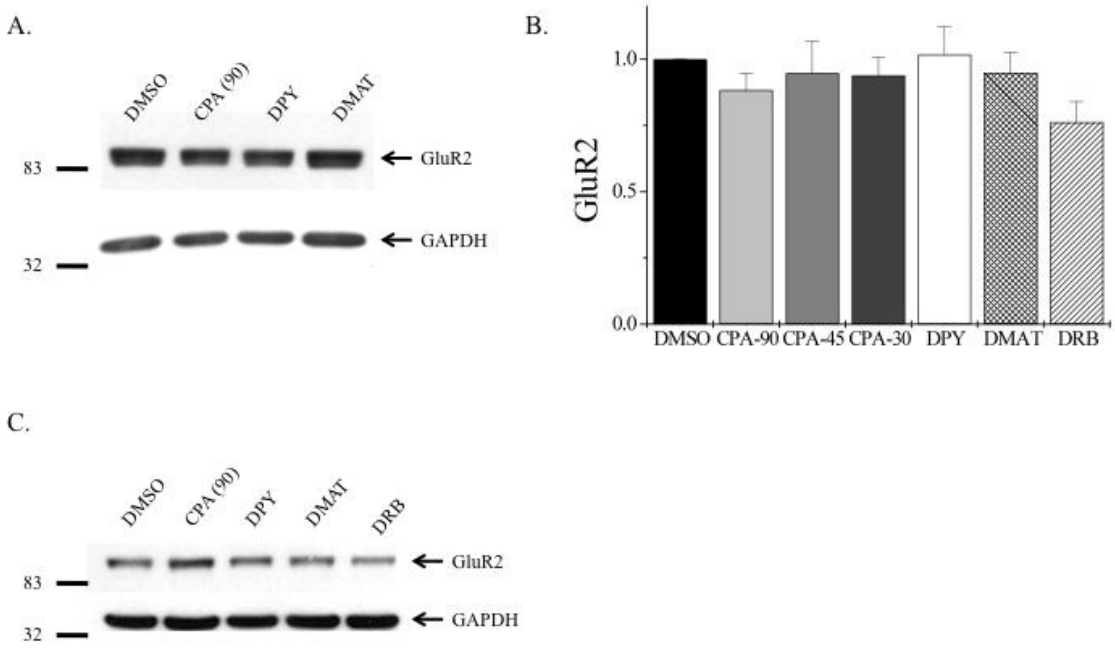

Figure 4.12. Application of the adenosine analogue CPA, ENT inhibition, or CK2 inhibition does not significantly change the localization of GluR2 in synaptosomal fractions. Hippocampal slices were exposed to either control DMSO, CPA (500nM), DPY (10μM), DMAT (5μM), or DRB (100μM) for 1.5hr (or 90, 45 or 30 min for CPA). Hippocampal homogenates were separated into synaptosomal fractions and used for Western Blot analysis. **A, C.** Application of CPA of the inhibition of CK2 or ENT did not cause a significant change in GluR2 localization in synaptosomal fractions. Representative Western blots showing GluR2 bands near 96 kDa. GAPDH protein bands (~37 kDa) used to normalize GluR2 signals. **B.** Quantitative representation of multiple Western blots showing CPa inhibition of CK2 or ENT did not significantly change GluR2 localization in the synaptosomal fraction. (p=0.2919, one-way ANOVA). Values are means ± SEM [DMSO (n=26), CPA (90 min) (n=17), CPA (45 min) (n=6), CPA (30 min) (n=3), DPY (n=17), DMAT (n=25), and DRB (n=13)].

4.4 Effect of temperature on ENT and AMPA receptor localization

It is known that the basal concentration of extracellular adenosine in rat hippocampal slices that are maintained at 21°C is approximately twice that at 32°C, thus it is apparent that the extracellular concentrations of adenosine in the brain are sensitive to temperature (Dunwiddie and Diao, 2000). It is suggested that this is due to a reduction in the function of ENT2, as ENT1 is less sensitive to temperature change (Dunwiddie and Diao, 2000). We therefore wanted to determine if the membrane localization of ENT1 or ENT2 was more or less sensitive to direct inhibition with varying temperatures.

As shown in Figure 4.13 and 4.14A, we found that there was a significant increase in the localization of the 60 kDa ENT1 protein in the membrane fraction of hippocampal tissue slices that were incubated, at room temperature (~21°C), with both 10nM (1.37321 +/- 0.1249; $p<0.001$) and 100nM (1.47084 +/- 0.0691; $p<0.001$) of the ENT1-specific inhibitor, NBTI, when compared to control DMSO. Additionally, we found that the membrane localization of the 50 kDa ENT1 was significantly enhanced after treatment with NBTI (10nM) (1.35987 +/- 0.17197) compared to the control ($p<0.01$), however there was no significant effect of NBTI (100nM) (1.08771 +/- 0.03414) compared to the control (Figure 4.14C), at room temperature.

We found that there was no significant change ($p>0.05$) in the membrane expression of the 50 or 60 kDa ENT1 protein in hippocampal slices that were incubated at 35°C (Figure 4.14B,D). There was also no significant difference ($p>0.05$) in the localization of either the 50 or 60 kDa ENT1 protein in the cytosolic fraction of the cell at either RT or 35°C (Figure 4.15 and Figure 4.16).

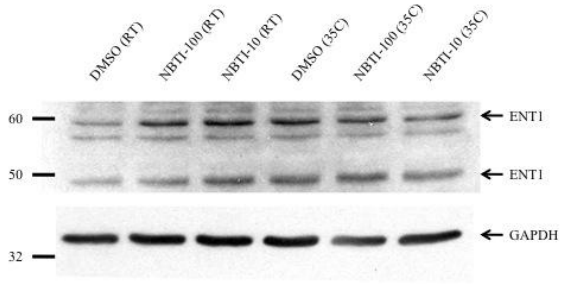

Figure 4.13. ENT1 membrane localization at RT and 35°C. *Top*. Representative Western blots showing that ENT1 band signals near 50 and 60 kDa are enhanced by the ENT1 inhibitor NBTI at RT in the membrane fraction of hippocampal slices. *Bottom.* GAPDH protein bands (~37 kDa) are used to normalize ENT1 signals.

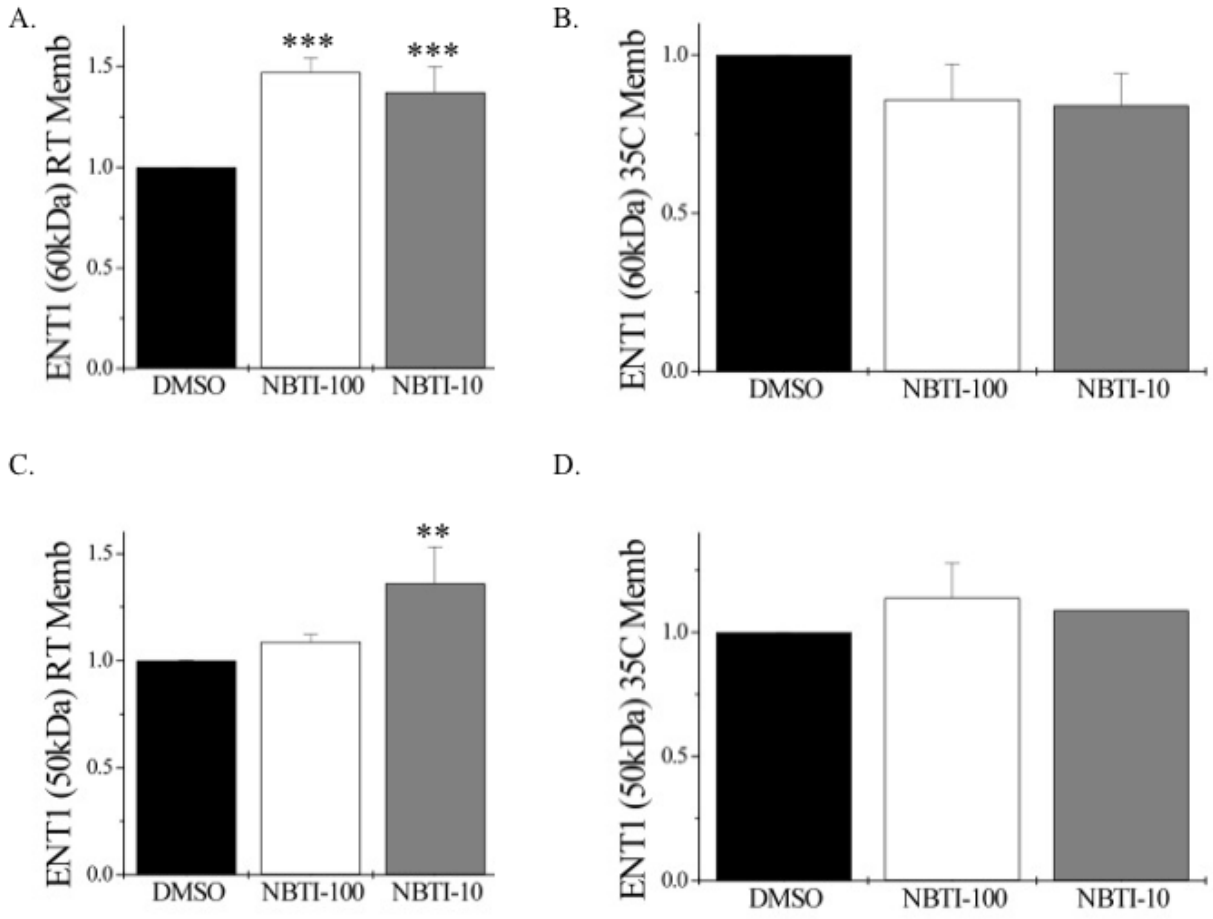

Figure 4.14. ENT1 inhibition causes an up-regulation of ENT1 surface localization at RT. Hippocampal slices were exposed to either control DMSO, NBTI (100nM) or NBTI (10nM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A, C.** Quantitative representation of multiple Western blots showing inhibition of ENT1 with NBTI enhanced the surface localization of the 50 and 60 kDa ENT1 protein at room temperature ($p<0.0001$, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=12), NBTI-100nM (n=4) and NBTI-10nM (n=4)] [ENT1 50 kDa- DMSO (n=16), NBTI-100nM (n=4), NBTI-10nM (n=3)]. **$p<0.01$, ***$p<0.001$ vs. control. **B, D.** Quantitative representation of multiple Western blots showing inhibition of ENT1 did not cause a significant change in the localization of the 50 ($p=0.2835$, on-way ANOVA) or 60 kDa ($p=0.3060$, one-way ANOVA) ENT1 protein at 35°C in membrane fraction. Values are means ± SEM [ENT1 60 kDa- DMSO (n=4), NBTI-100nM (n=4), and NBTI-10nM (n=4)] [ENT1 50 kDa- DMSO (n=6), NBTI-100nM (n=3), and NBTI-10nM (n=2)].

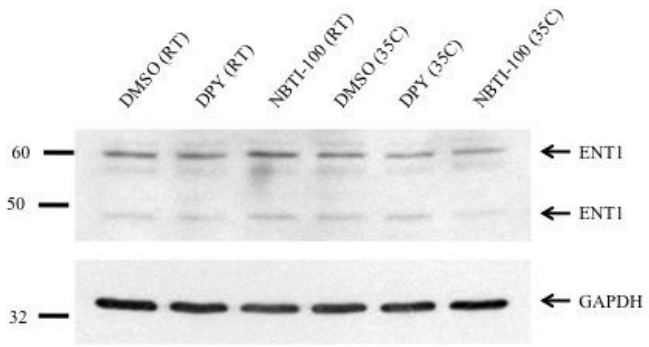

Figure 4.15. Cytosolic localization of ENT1 at RT and 35°C. *Top.* Representative Western blots showing that, in the cytosolic fraction of cells from hippocampal tissue slices, ENT1 band signals near 50 and 60 kDa do not significantly change after treatment with ENT inhibitors NBTI or DPY at RT or 35°C. *Bottom.* GAPDH protein bands (~37 kDa) are used to normalize ENT1 signals.

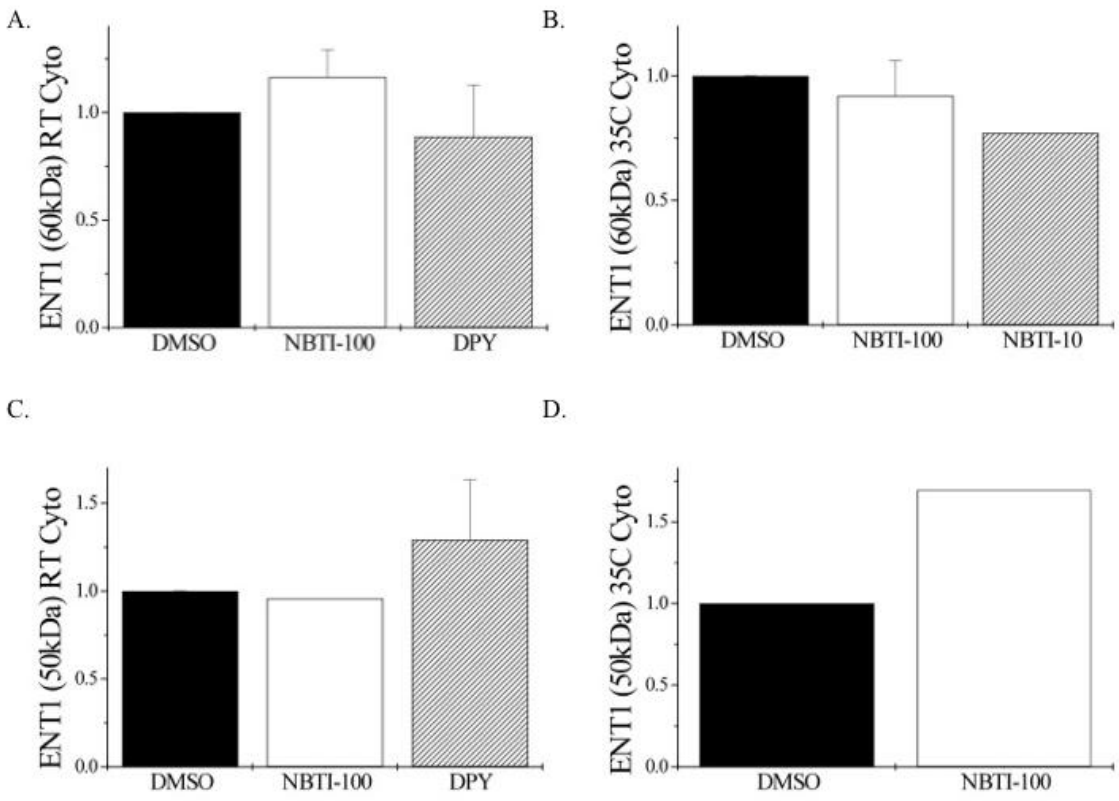

Figure 4.16. ENT inhibition does not affect the cytosolic localization of ENT1 at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A, C.** Quantitative representation of multiple Western blots showing inhibition of ENTs does not significantly affect the localization of the 50 and 60 kDa ENT1 protein at room temperature ($p=0.5237$; $p=0.3487$, respectively, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=10), NBTI-100nM (n=5) and DPY (n=5)] [ENT1 50 kDa- DMSO (n=7), NBTI-100nM (n=2) and DPY (n=5)]. **B, D.** Quantitative representation of multiple Western blots showing inhibition of ENT1 did not cause a significant change in the localization of the 50 or 60 kDa ENT1 protein at 35°C in membrane fraction ($p=0.5870$; $p=0.6722$, respectively, one-way ANOVA). Values are means ± SEM [ENT1 60 kDa- DMSO (n=5), NBTI-100nM (n=5), and NBTI-10nM (n=2)] [ENT1 50 kDa- DMSO (n=2) and NBTI-100nM (n=2)].

In this set of experiments, only the ENT2 protein at 50 kDa was detectible, perhaps due to an insufficient exposure time during the ECL (Figure 4.17A,C). Nevertheless, analogous to what was seen in the localization of the 50 kDa ENT2 protein with CK2 inhibition, we found that, compared to the control, NBTI (10nM), NBTI (100nM), and DPY (10μM) did not cause any significant changes ($p>0.05$) in the membrane localization of ENT2 at RT or 35°C (Figure 4.17B, D). Again, there were no significant changes ($p>0.05$) in the localization of ENT2 in the cytosolic fraction at RT, or at 35°C in the cytosolic fractions (Figure 4.18).

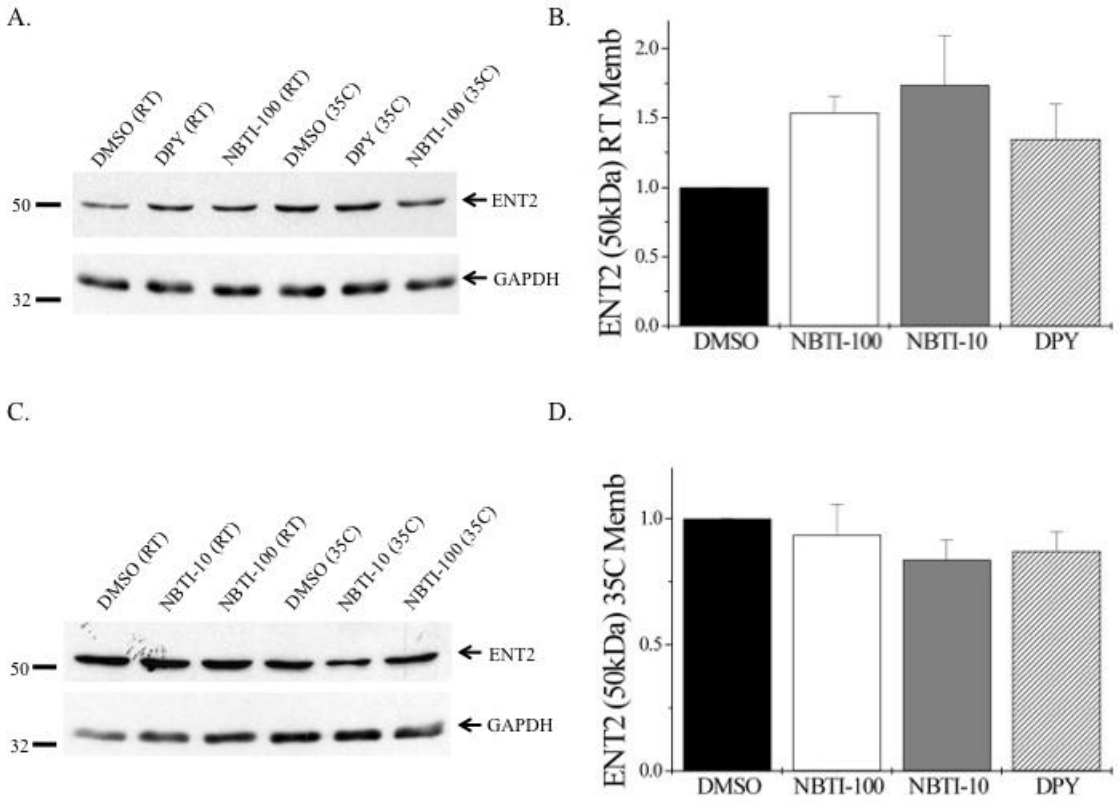

Figure 4.17. Membrane localization of ENT2 in the hippocampus is not significantly affected by ENT inhibition at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A, C.** Representative Western blots showing that ENT2 band signals at 50 kDa, in the hippocampal membrane fraction, are not changed by ENT inhibitors at RT or 35°C. GAPDH protein bands (~37 kDa) are used to normalize GluR1 signals. **B, D.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not significantly affect surface localization of the 50 kDa ENT2 protein at room temperature ($p=0.0618$, one-way ANOVA; B) or 35°C ($p=0.0897$, one-way ANOVA; D). Values are means ± SEM [ENT2 50 kDa RT- DMSO (n=16), NBTI-100nM (n=5), NBTI-10nM (n=4) and DPY (n=12)] [ENT2 50 kDa 35°C- DMSO (n=11), NBTI-100nM (n=5), NBTI-10nM (n=4), DPY (n=6)].

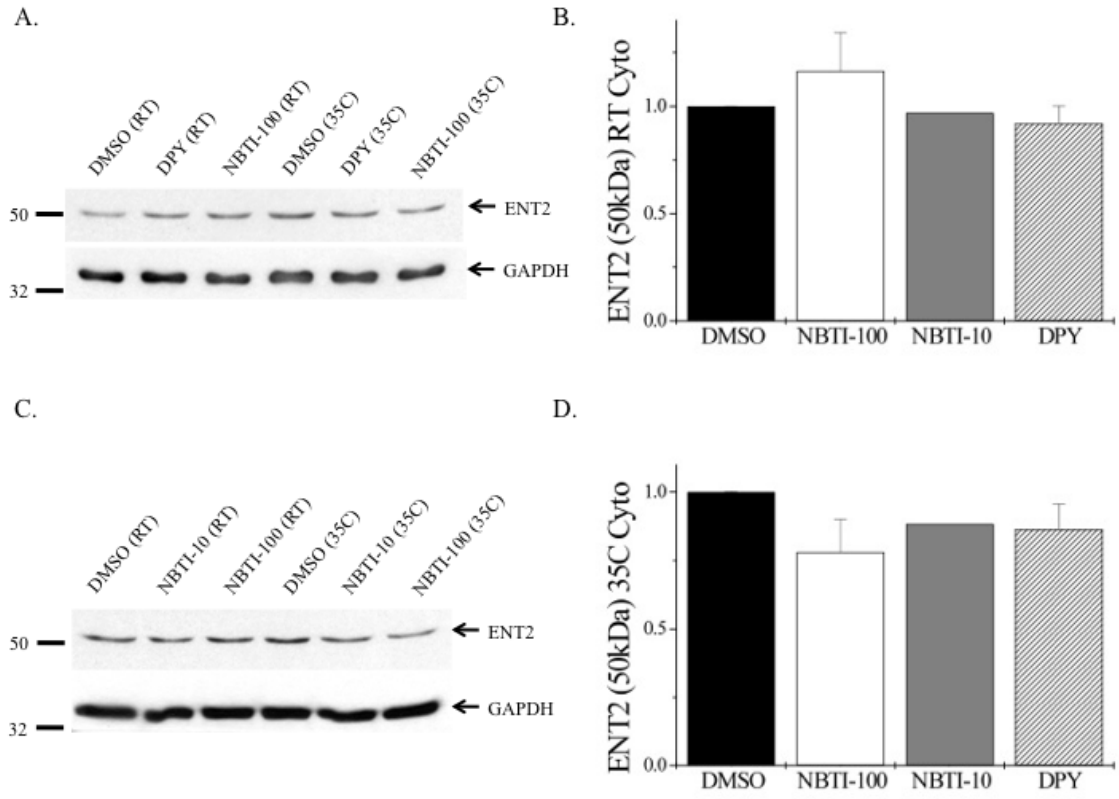

Figure 4.18. Cytosolic localization of ENT2 in the hippocampus is not significantly affected by ENT inhibition at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and used for Western Blot analysis. **A, C.** Representative Western blots showing that ENT2 band signals at 50 kDa, in the hippocampal cytosolic fraction, are not changed by ENT inhibitors at RT or 35°C. GAPDH protein bands (~37 kDa) are used to normalize GluR1 signals. **B, D.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not significantly affect cytosolic localization of the 50 kDa ENT2 protein at room temperature ($p=0.2644$, one-way ANOVA; B) or 35°C ($p=0.3050$, one-way ANOVA; D). Values are means ± SEM [ENT2 50 kDa RT- DMSO (n=12), NBTI-100nM (n=4), NBTI-10nM (n=2), DPY (n=10)] [ENT2 50 kDa 35°C- DMSO (n=5), NBTI-100nM (n=4), NBTI-10nM (n=2), DPY (n=4)].

In the membrane fraction of the cell, direct ENT inhibition with NBTI at RT showed a trend toward the up-regulation of GluR1 and GluR2 surface localization, whereas DPY showed a trend the down-regulation of GluR1 and GluR2 surface localization (Figures 4.19A, C and 4.21A, C); however, this change was not significant ($p>0.05$) compared to the control (Figure 4.19B, 4.21B). Additionally, there were no significant changes ($p>0.05$) among the treatment groups for GluR1 and GluR2 cytosolic fractions at room temperature, and there were no significant changes ($p>0.05$) in the localization of GluR1 or GluR2 at 35°C (Figures 4.19-4.22).

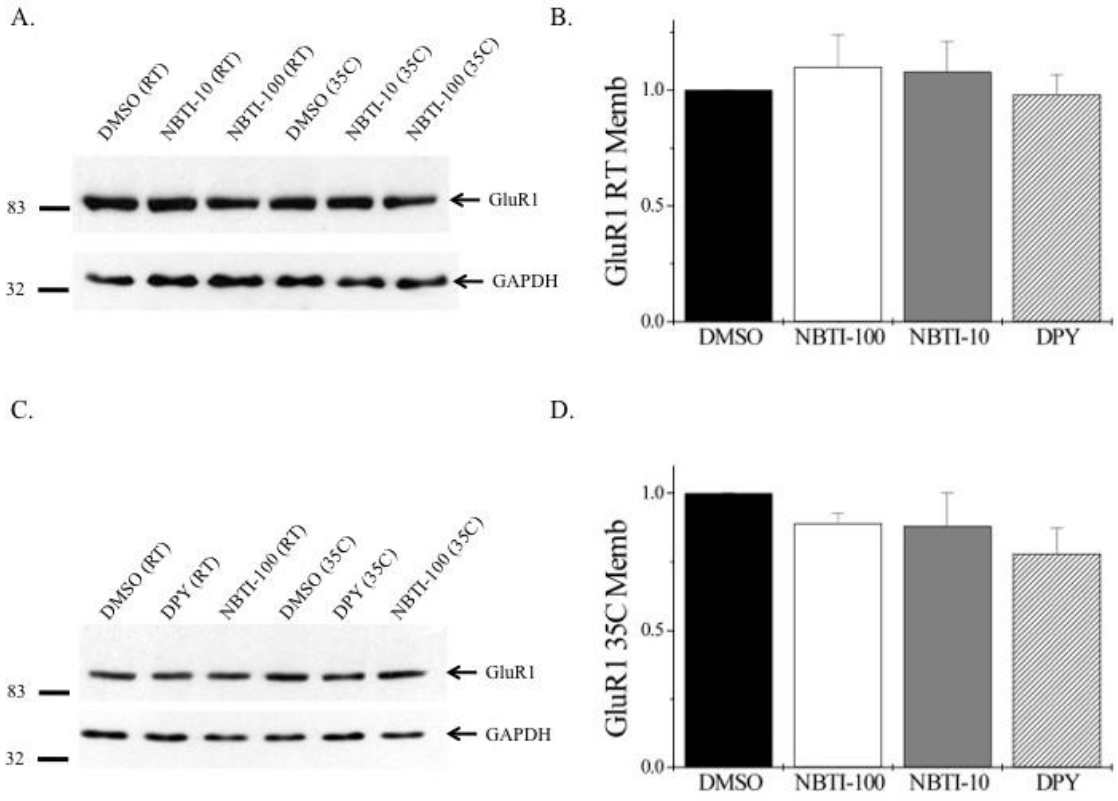

Figure 4.19. Inhibition of ENTs does not cause a reduction of GluR1 in membrane fractions at RT or 35°C. Hippocampal slices were exposed to control DMSO aCSF, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and the membrane fraction was used for Western Blot analysis. **A, C.** Representative Western blots showing that GluR1 band signals at RT and 35°C in the membrane fraction, near 106 kDa, are not changed by ENT inhibitors. GAPDH protein bands (~37 kDa) are used to normalize GluR1 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not cause a significant change in GluR1 surface localization at RT ($p=0.7358$, one-way ANOVA). Values are means ± SEM [DMSO (n=21), NBTI-100nM (n=5), NBTI-10nM (n=5) and DPY (n=17)]. **D.** Quantitative representation of multiple Western blots showing that there was no significant change in the membrane localization of GluR1 by ENT inhibition at 35°C. ($p=0.1483$, one-way ANOVA). Values are means ± SEM [DMSO (n=8), NBTI-100nM (n=5), NBTI-10nM (n=5) and DPY (n=4)].

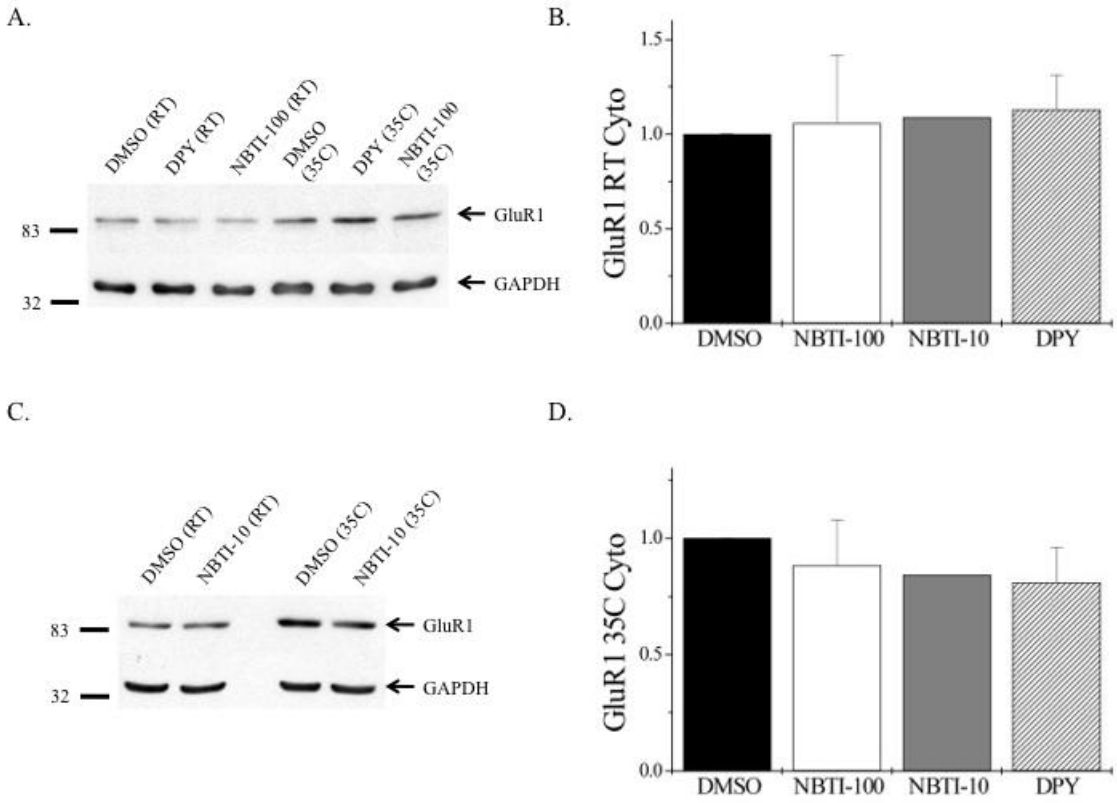

Figure 4.20. Inhibition of ENTs does not change GluR1 localization in cytosolic fractions at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and the membrane fraction was used for Western Blot analysis. **A, C.** Representative Western blots showing that GluR1 band signals at RT and 35°C in the cytosolic fraction, near 106 kDa, are not changed by ENT inhibitors. GAPDH protein bands (~37 kDa) are used to normalize GluR1 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not cause a significant change in GluR1 cytosolic localization at RT ($p=0.7977$, one-way ANOVA). Values are means ± SEM [DMSO (n=8), NBTI-100nM (n=3), NBTI-10nM (n=2) and DPY (n=4)]. **D.** Quantitative representation of multiple Western blots showing that there was no significant change in the cytosolic localization of GluR1 by ENT inhibition at 35°C. ($p=0.6154$, one-way ANOVA). Values are means ± SEM [DMSO (n=6), NBTI-100nM (n=3), NBTI-10nM (n=2) and DPY (n=5)].

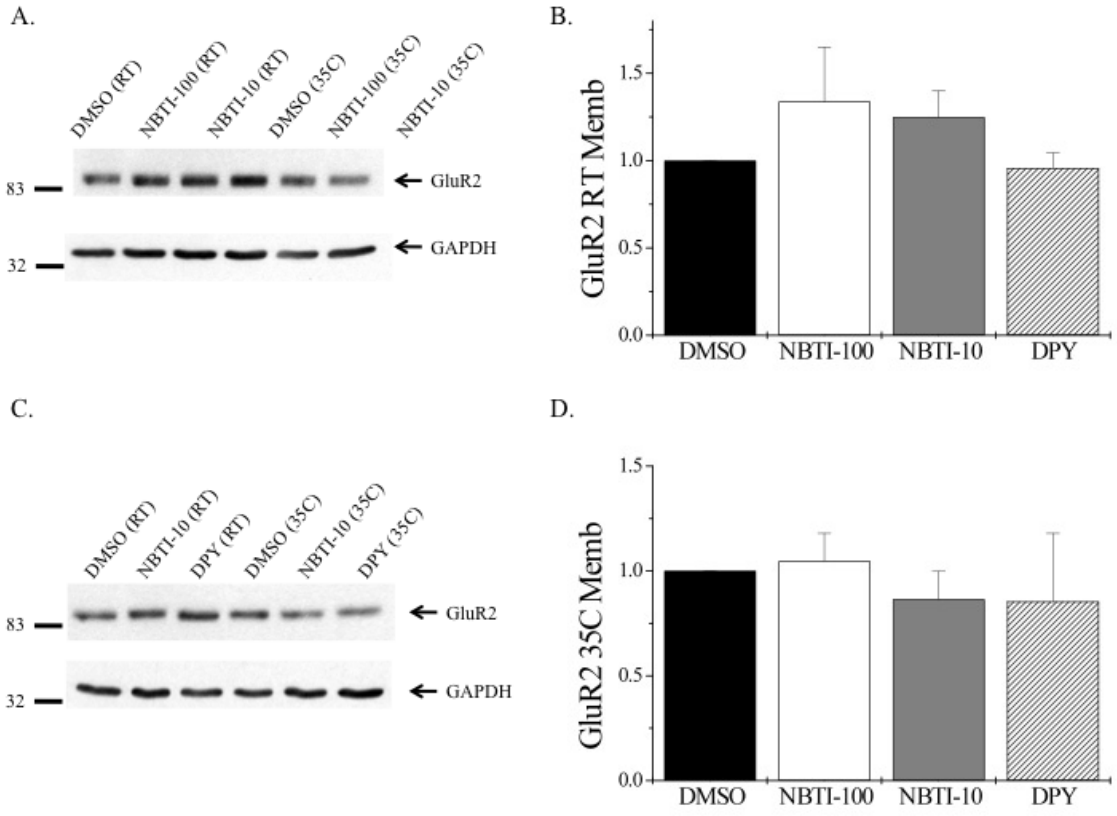

Figure 4.21. Inhibition of ENTs does not cause a reduction in GluR2 localization in membrane fractions at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and the membrane fraction was used for Western Blot analysis. **A, C.** Representative Western blots showing that GluR2 band signals at RT and 35°C in the membrane fraction, near 96 kDa, are not changed by ENT inhibitors. GAPDH protein bands (~37 kDa) are used to normalize GluR2 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not cause a significant change in GluR2 surface localization at RT ($p=0.0518$, one-way ANOVA). Values are means ± SEM [DMSO (n=23), NBTI-100nM (n=6), NBTI-10nM (n=7) and DPY (n=16)]. **D.** Quantitative representation of multiple Western blots showing that there was no significant change in the membrane localization of GluR2 by ENT inhibition at 35°C. ($p=0.7436$, one-way ANOVA). Values are means ± SEM [DMSO (n=10), NBTI-100nM (n=6), NBTI-10nM (n=7) and DPY (n=3)].

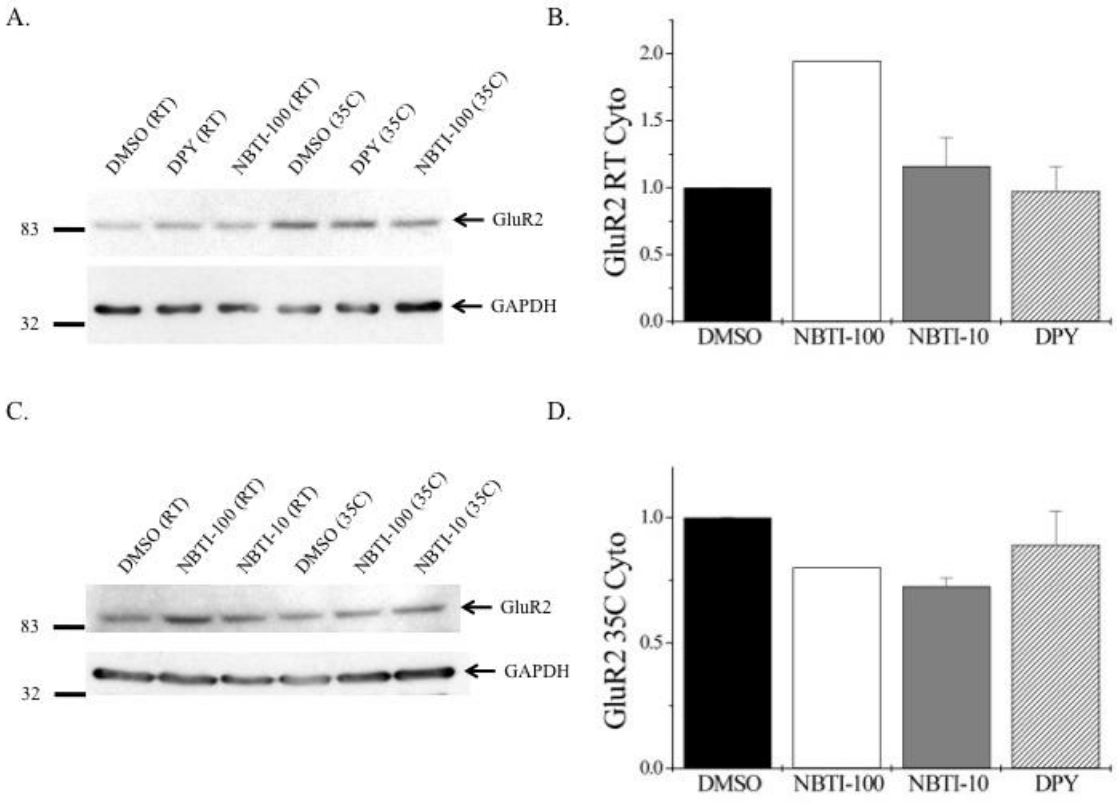

Figure 4.22. Inhibition of ENTs does not change GluR2 localization in cytosolic fractions at RT or 35°C. Hippocampal slices were exposed to either control DMSO, NBTI (100nM), NBTI (10nM) or DPY (10μM) for 1.5hr at either RT or 35°C. The membrane and cytosolic fractions of hippocampal homogenates were separated by centrifugation and the membrane fraction was used for Western Blot analysis. **A, C.** Representative Western blots showing that GluR2 band signals at RT and 35°C in the cytosolic fraction, near 96 kDa, are not changed by ENT inhibitors. GAPDH protein bands (~37 kDa) are used to normalize GluR2 signals. **B.** Quantitative representation of multiple Western blots showing inhibition of ENTs did not cause a significant change in GluR2 cytosolic localization at RT ($p=0.6847$, one-way ANOVA). Values are means ± SEM [DMSO (n=12), NBTI-100nM (n=2), NBTI-10nM (n=3) and DPY (n=8)]. **D.** Quantitative representation of multiple Western blots showing that there was no significant change in the cytosolic localization of GluR2 by ENT inhibition at 35°C. ($p=0.1060$, one-way ANOVA). Values are means ± SEM [DMSO (n=4), NBTI-100nM (n=2), NBTI-10nM (n=3) and DPY (n=3)].

CHAPTER 5

GENERAL DISCUSSION

Equilibrative nucleoside transporters ENT1 and ENT2 are expressed ubiquitously in plasma membranes of mammalian cells, and are distributed throughout the central nervous system, including the hippocampus (Jennings et al., 2001). Little is known about the how the localization of these transporters is regulated but a variety of reports suggest that ENTs can be modified by kinase-dependent pathways (Coe et al., 1996; Coe et al., 2002; Kong et al., 2004; Bone et al., 2007). ENT1 and ENT2 have a large intracellular loop joining transmembrane domains 6 and 7, which contains sites for phosphorylation by CK2 (Kiss et al., 2000; Handa et al., 2001; Stolk et al., 2005). Thus, the presence of these phosphorylation sites suggests that the transporter activity and/or membrane localization of ENTs could be subject to modulation by CK2.

5.1 Membrane localization of ENTs

ENT1 and ENT2 are expressed on the plasma membrane (Alanko et al., 2006) of neuronal and non-neuronal cells (Anderson et al., 1999a; Anderson et al., 1999b; Parkinson et al., 2005) and are localized in regions of the brain known to contain adenosine receptors (Anderson et al., 1999a; Anderson et al., 1999b; Jennings et al., 2001), emphasizing the importance of these transporters in the regulation of adenosine tone. Here we confirm the presence of ENT1 and ENT2 in membrane fraction of cells prepared from rat hippocampal tissue, and show that these transporter proteins are located at 50 and 60 kDa, and that there is also a high concentration of these proteins at 83 kDa, previously described to be ENT protein dimers (Reyes et al.).

Hippocampal membrane fractions were prepared in this study to determine whether the membrane localization of these transporters could be altered by CK2 inhibition. Here, we have shown that incubation of hippocampal tissue slices with CK2 inhibitors resulted in a significant decrease in ENT1 (33-43%; $p<0.01$) and ENT2 (32-56%; $p<0.001$) protein density in hippocampal membrane fractions, inferred as a decrease in membrane localization of the 60 kDa

transporter protein. This data implies that ENT1 and ENT2 protein localization in hippocampal tissue may normally be regulated by CK2 activity.

It was not confirmed whether CK2 inhibition prevented the phosphorylation of ENT1 and ENT2 at CK2 consensus sites in this study, however the findings presented here are in agreement with those of Bone et al. 2007, who found that inhibition of CK2 activity with TBB produced a reduction in the number of functional ENT1 proteins at the level of the plasma membrane. Deletion of Ser-254 from mouse ENT1 also renders ENT1 levels insensitive to TBB-mediated inhibition of CK2 (Bone et al., 2007). Together, this evidence strongly implies that Ser-254 is phosphorylated by CK2 in ENT1. However, Stolk et al. 2005 suggests that the inhibition of CK2 activity has a dual effect on ENT1, as they observed transient inhibition of ENT1 transcription followed by a delayed post-transcriptional increase in the number of transporters functioning at the plasma membrane, after the induction of the catalytically inactive CKα' in osteosarcoma cells. They also observed a reduction in the plasma membrane localization of ENT2, which was parallel to a reduction in the level of mENT2 mRNA (Stolk et al., 2005). Here we found no significant change ($p>0.05$) in the cytosolic localization of ENT1 and ENT2 (Figures 4, 6), however, because the relative concentration of these transporters in the membrane and cytosolic fractions were not directly compared, it is difficult to determine whether the ENTs were undergoing trafficking between the plasma membrane and intracellular stores, as seen with glucose transporters (Baldwin et al., 1995), or whether they were being rapidly endocytosed and degraded. Thus, the mechanism by which CK2-mediated phosphorylation regulates the membrane localization of ENT1 and ENT2 requires further investigation, and it may be beneficial to employ more precise techniques, such as a cell surface biotinylation assay (Gallyas et al., 2003), for future study to confirm the membrane trafficking of these proteins.

The concentration of ENT proteins detected near 50 kDa was greater than that detected at 60 kDa, however inhibition of CK2 function did not cause significant changes in the concentration of these proteins; only the 60 kDa proteins were significantly altered by CK2 inhibition. Although it is not recognized in rat or human tissue, a splice variant of ENT1 has been identified in murine tissue (Kiss et al., 2000; Handa et al., 2001). The shorter of the two splice variants, mENT1a, has greater expression in brain tissue than the larger variant, mENT1b (Bone et al., 2007). It has also been shown that the number of mENT1b transporters operating at the level of the plasma membrane are down regulated by CK2 inhibition, while mENT1a are not

(Bone et al., 2007). It is possible that there also exists a rat ENT1 splice variant that has not yet been identified. The presence of a rat splice variant may help to account for the lack of susceptibility to regulation by CK2-mediated phosphorylation observed in the 50 kDa proteins relative to that of the 60 kDa proteins; however this is purely hypothetical and needs further experimental confirmation. Alternately, it is likely that the 50 kDa proteins represent ENT proteins that have undergone less post-translational modification compared to the 60 kDa proteins, for example, less N-linked glycosylation. Although ENT proteins have been detected at various molecular weights, it is apparent from the number of non-specific protein bands in the ENT1 and ENT2 Western blots that the specificity of the ENT antibodies is a caveat of this study. While an antigenic peptide for the ENT antibodies was used as an attempt to address this issue, the results were inconclusive as the antibodies and peptides used were extremely sensitive to the blotting procedure, and optimization of the signal was not possible.

To ensure that the effect of CK2 inhibition on ENT localization was not due to the activity of other intracellular proteins that might modify the localization and/or function of ENTs, a variety of CK2 inhibitors were used, at concentrations that have been shown by a number of other groups to selectively inhibit CK2 (Lieberman and Mody, 1999; Sarno et al., 2001; Pinna, 2002; Pagano et al., 2004). Each of the CK2 inhibitors used in this thesis are derivatives of the nucleoside analogue 5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (DRB). DRB has a K_i value of 23 μM (Cozza et al.), and works by occupying the hydrophobic pocket adjacent to the ATP/GTP binding site in CK2 and competitively inhibits the binding of these phosphate donors (Cozza et al.; Sarno et al., 2001; Sarno et al., 2002; Sarno et al., 2005; Mazzorana et al., 2008). DRB is somewhat of a dirty drug, as it is known to inhibit topoisomerase, cellular and viral RNA and result in relatively high cytotoxicity, however, the structure of this molecule has been progressively optimized and allowed for the development of derivatives that better interact with the ATP binding pocket, including both TBB and DMAT with K_i values of 0.40 and 0.04 μM, respectively (Cozza et al.; Sarno et al., 2005). Other protein kinases generally inhibited by CK2 ligands include PIM1, 2, 3, DYRK1a, 2, 3 and in some cases GSK3β and CDK2 (Cozza et al.). However, when tested against a panel of 32 kinases other than CK2, DMAT and TBB affected only DYRK1a, with similar efficacy (Sarno et al., 2005). Furthermore, all of the CK2 inhibitors produced similar effects, giving indication that CK2 was selectively inhibited. However, it cannot be guaranteed that CK2 inhibition did not change the

activity of its other substrates that are known to modify ENT function and/or localization (Mead et al., 2003; Soler et al., 2003; Castelli et al., 2004; Sperlagh et al., 2004). For instance, PKC is a known substrate for CK2 (Blanquet, 2000), and has been shown to modify the activity and expression of ENTs under some circumstances (Chaudary et al., 2004).

Here we have confirmed that the membrane localization of ENT1 and ENT2 in the rat hippocampus is sensitive to changes in CK2 activity, such that CK2 inhibition causes a reduction in the concentration ENT1 and ENT2 in the membrane fraction. Although the exact mechanism for these changes is unknown, it is evident that these changes in ENT localization may potentially alter the level of extracellular adenosine, thus altering adenosinergic signaling, and possibly the susceptibility of hippocampal cells to excitotoxicity during ischemic events.

5.2 ENT localization at neuronal synapses

Several groups have described the localization of ENT1 and ENT2 mRNA and protein in neurons and astrocytes (Anderson et al., 1999a; Anderson et al., 1999b; Parkinson et al., 2005). Additionally, CK2 activity is associated with synaptosomes (Girault et al., 1990), indicating that CK2 is present at substantial levels in nerve terminals (Blanquet, 2000). To address the issue of having a mixture of neuronal and non-neuronal cells in our hippocampal lysate, synaptosomes were prepared in an attempt to separate the effect of CK2 inhibition in these different cell types. The data presented in this study indicates there was no significant ($p>0.05$) change in the localization of ENT1 or ENT2 in neuronal synapses after treatment with CK2 inhibitors (Figures 4.6 and 4.7). These findings differ from that observed in the membrane fraction of the cells prepared from hippocampal slices (Figures 4.1 and 4.3). While the activity of CK2 in the brain is predominantly neuronal, it is also associated with glia (Girault et al., 1990; Parkinson et al., 2005). It is therefore possible that the overall reduction in ENT1 and ENT2 surface localization observed in the membrane fraction of the hippocampal lysates may be attributed to changes in ENT1 and ENT2 at extra-synaptic locations and in non-neuronal tissue. Treatment with the adenosine analogue CPA or the direct ENT inhibitor DPY, which were used to simulate an increase in extracellular adenosine tone, also failed to cause any significant change ($p>0.05$) in the localization of ENT1 or ENT2 in the synaptosomal preparations. However, ENT proteins have been identified in cortical synaptosomes, where adenosine transport is reduced in the

presence of DPY (Parkinson et al., 2009), yet this does not necessarily mean a reduction in localization at the synapse. Another, and perhaps more effective, means to separate the glial cell contribution in our cell lysates would have been to use cultured hippocampal neurons and glia paired with immunocytochemistry to localized these receptors in each cell type. While this technique was attempted, it still needs to be optimized in order to obtain reliable results.

5.3 ENTs are physically associated with proteins involved in synaptic transmission

There have been a few studies where the distribution of ENT1 and ENT2 mRNA or protein has been observed in regions of the rat and human brain that are also known to contain adenosine receptors (Anderson et al., 1999a; Anderson et al., 1999b; Jennings et al., 2001). Recently, our research group has found evidence that A1 adenosine receptors are physically associated with GluR2 AMPA receptors (Z. Chen, unpublished data), and here, via immunoprecipitation, we show that ENT1 and ENT2 are physically associated with the GluR1and GluR2 AMPA receptor subunits in whole cell hippocampal lysates (Figure 4.8). Together, this evidence suggests that these molecules might function together in a larger molecular complex, linking the regulation of adenosine signaling and AMPA receptor subunit redistribution. The direct signaling mechanism linking these molecules is not known; however, mitogen activated protein kinases (MAPKs) may be a key player in this proposed signaling complex. Two subfamilies of MAPKs, p38 MAPK and c-Jun N-terminal kinase (JNK), have been shown to be an important link between adenosine A1 receptor activation and the inhibition of synaptic transmission pre-synaptically (Brust et al., 2006; Brust et al., 2007). A role for p38 MAPK and JNK has been implicated in the redistribution of GluR1 and GluR2 receptors (Boudreau et al., 2007). Additionally, a link between nucleoside transport activity and the MAPK pathway has been proposed (Sperlagh et al., 2004), and CK2 has been reported to modify MAPK signaling cascades via interaction with both protein phosphatase 2A and MAPK phosphatase 3 (Lebrin et al., 1999; Perez and Avila, 1999; Castelli et al., 2004).

5.4 Inhibition of CK2 alters AMPA receptor localization

GluR1 and GluR2 contain many residues that are consensus sites for phosphorylation by various protein kinases, and it has been shown that phosphorylation of these subunits is an important type of post-translational modification that regulates AMPA receptor function (Santos et al., 2009). However, although CK2 is known to regulate NMDA glutamate channel function in the hippocampus, it has not been shown to regulate AMPA receptor function (Lieberman and Mody, 1999; Kimura and Matsuki, 2008). So, while it has been shown that nucleoside transporters critically modulate glutamatergic synaptic transmission (Ackley et al., 2003), and that adenosine uptake inhibitors, such as DPY, cause a decrease in extracellular field potential recordings (Dunwiddie and Diao, 2000), the effects of CK2 inhibition on AMPA receptor trafficking have not yet been extensively explored.

Here, we report that treatment of hippocampal tissues with DMAT, TBB and DRB caused 29-35% reduction in the surface localization of the GluR1 AMPA receptor subunit and a 40-56% reduction in the surface localization of the GluR2 AMPA receptor subunit. Thus, it is possible that CK2 inhibitors contribute to a reduction in the membrane localization of GluR1 and GluR2-containing AMPA receptors by enhancing adenosine A1 receptor activation, via a reduction in ENT1 and ENT2 at the plasma membrane and accumulation of extracellular adenosine tone, as summarized in Figure 5.1. Indeed, ischemic insults, which rise extracellular levels of adenosine, are thought to cause remodeling of AMPA receptor subunits and a long-lasting switch in AMPA receptor phenotype, from GluR2-containing to GluR2-lacking receptors (Liu and Zukin, 2007). Although the reduction in the Ca^{2+}-permeable GluR1 subunit seen here was not anticipated, it is probable that there was a simultaneous reduction in GluR1 and GluR2 AMPA receptor subunits, as they often exist together as heteromeric AMPA receptors, which are known to follow the same sorting as GluR2 homomers (Lee et al., 2004).

While we saw that there was a reduction in the amount of GluR1 and GluR2 protein expressed in the membrane fraction of hippocampal cell lysates, there was no apparent increase in the amount of GluR1 or GluR2 in the cytosol. It is known that there are endogenous mRNAs encoding these AMPA receptor subunits that are localized in proximal and distal dendrites of hippocampal neurons (Grooms et al., 2006), suggesting that the composition and abundance of receptors is regulated by local synthesis (Santos et al., 2009). Additionally, CK2 has many

substrates that are known transcription factors, which are also involved in the regulation of synaptic transmission (Blanquet, 2000). So, while it is possible that CK2 inhibition caused a reduction in the transcription of these AMPA receptor subunits, based on the time course of the treatments in this study, it is unlikely that the changes membrane localization seen here are due to a transcriptional effect of CK2.

Recent studies show that after high frequency stimulation there is a reversible increase in GluR1 and GluR2 protein levels in synaptosomes isolated from the hippocampal CA1 region (Lu et al., 2002; Santos et al., 2009). Additionally, it has been shown that CK2 activity is associated with synaptosomes (Girault et al., 1990; Blanquet, 2000) and that CK2 activity is transiently increased after high frequency stimulation (Charriaut-Marlangue et al., 1991). Thus, inhibition of CK2 may cause a reduction in the GluR1 and GluR2 AMPA receptor subunits at the synapse, similar to what was seen in the whole hippocampal membrane fraction. However, densitometry analysis of GluR1 and GluR2 at the neuronal synapse revealed that there was no significant change in the localization of either GluR1 or GluR2 in this region of the cell after incubation with CK2 inhibitors ($p>0.05$) or the direct ENT inhibitor, DPY ($p>0.05$), which is expected to increase extracellular adenosine tone. The adenosine analogue CPA also failed to cause a significant change ($p>0.05$) in the localization of GluR1 or GluR2 in synaptosomal fractions, which, based on previous work from our lab, would be expected to produce a reduction in the membrane localization of these proteins. It is possible however, that the changes in GluR1 and GluR2 localization that are seen in the membrane fraction are occurring at extra-synaptic sites, and although AMPA receptors are considered neuronal receptors; they have been detected in glial cells (Santos et al., 2009), thus it is possible that astrocytic GluR1 and GluR2 may contribute to the observed changes in membrane-localized AMPA receptors subunits.

It is already known that the level of CK2 activity is reduced after periods of brief ischemia (Hu and Wieloch, 1993), and although not presented in this thesis, our lab has shown that CK2 inhibition in the presence of hypoxia causes a significant reduction in the membrane localization of ENT1. Furthermore, it was found that CK2 inhibition potentiates the internalization of GluR2 during hypoxia (Z. Chen, unpublished data). These data suggest that a reduction in CK2 activity during hypoxia potentiates the changes in ENT1 and GluR2 localization seen under normoxic conditions, and that this may contribute to enhanced neuronal injury during ischemic attacks. Therefore, the regulation of CK2 activity may be an important

target for neuroprotective strategies. Consistent with our observations, another study, of human umbilical vein endothelium exposed to a hypoxic environment, has shown a reduction in hENT1 function but an increase in extracellular adenosine concentration (Casanello et al., 2005). However, chronic hypoxia has been suggested to cause a down-regulation of rENT1 (Kobayashi and Millhorn, 2001) and mENT1 mRNA which is dependent on the down-regulation of PKCε (Chaudary et al., 2004). Thus, it is possible that regulatory mechanisms other than the inhibition of CK2-mediated phosphorylation of ENTs contribute to the hypoxia-induced reduction in ENT1 localization seen here.

It is well known that the extracellular level of adenosine is enhanced during both metabolic insults such as ischemia as well as with the inhibition of ENTs, and it is widely accepted that enhanced extracellular adenosine levels produce neuroprotective benefits (Cunha, 2001; Dunwiddie and Masino, 2001; Olsson et al., 2004; Fredholm et al., 2005). However, unlike the A1 receptor, activation of the A2a receptors may contribute to neuronal damage, as the antagonism of A2a receptors has been shown to offer neuroprotection in ischemic and excitotoxic models (Fredholm et al., 2005). It is also known that adenosine stimulates the release of astrocytic glutamate via an A2a adenosine receptor/PKA pathway (Nishizaki, 2004). Thus, it is possible that ENT inhibition may result in enhanced neuronal excitotoxicity via A2a receptors. In the hippocampus, A1 receptor density is reduced with age, whereas the A2a receptor density is increased (Cunha et al., 1995). Additionally, A2a receptor-mediated facilitation of hippocampal synaptic transmission is enhanced with age, whereas A1 receptor signaling becomes less efficient (Lopes et al., 1999b; Cunha, 2001; Rebola et al., 2003). This suggests that with age, when the risk of ischemic neuronal injury is increased, the A2a adenosine receptor-mediated pathway may become the dominant pathway for adenosine signaling. Therefore, increases in extracellular adenosine may cause some neuronal damage in an A2a receptor-dependent mechanism.

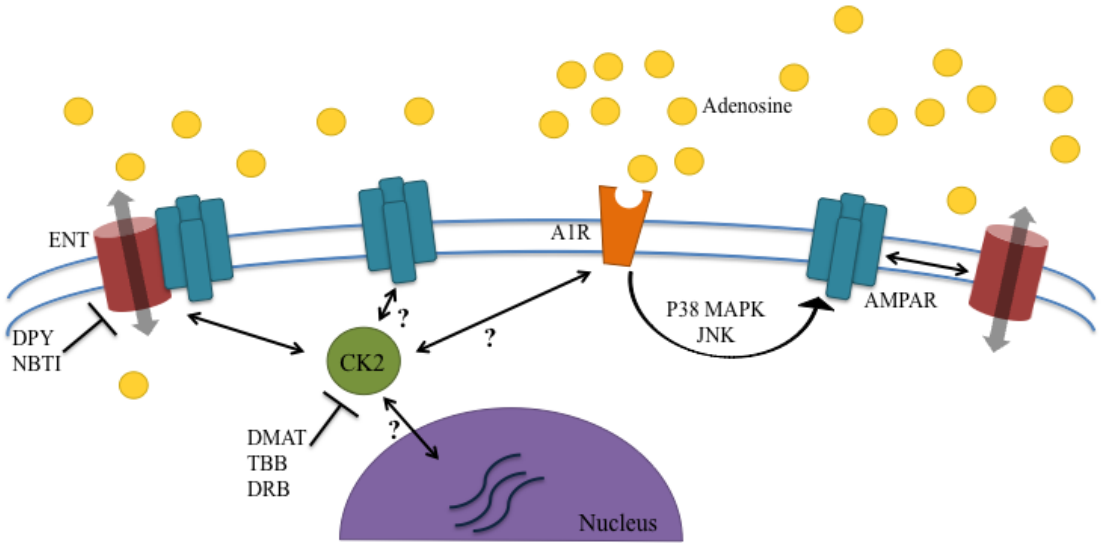

Figure 5.1. Proposed model for the CK2-mediated regulation of ENT1, ENT2 and AMPAR subunits GluR1 and GluR2 surface localization in the rat hippocampus. Potential coupling between ENTs and GluR subunits may be intimately linked with the adenosine A1 receptor (A1R) signaling. Phosphorylation of ENTs by protein kinase CK2, promotes ENT surface localization. Treatment with CK2 inhibitors DMAT, TBB, or DRB leads to reduction in ENT surface localization, and subsequent rise in extracellular adenosine tone. Extracellular adenosine acts on A1Rs, causing the activation of p38 MAPK and JNK, and in turn, a reduction in the surface localization of GluR1 and GluR2 AMPAR subunits. Our evidence also suggests a physical interaction between ENTs and GluR1 and GluR2, thus these AMPA receptors may be internalized with ENTs in a protein complex. Treatment with ENT inhibitors DPY and NBTI leads to increased adenosine tone extracellularly, depending on the extent to which they block adenosine efflux/influx, and therefore may cause AMPAR subunit redistribution. The mechanism by which CK2-mediated phosphorylation regulates the membrane localization of ENT1 and ENT2 requires further investigation, as we could not determine whether or not CK2 inhibition had any affect on ENT transcription, or whether it was a post-translational effect.

5.5 ENT and AMPA receptor localization is temperature sensitive

It has been suggested that ENTs are sensitive to changes in temperature (Dunwiddie and Diao, 2000); therefore we wanted to determine if the membrane localization of ENT1 or ENT2 was more or less sensitive to direct inhibition with varying temperatures. Here, we report a significant increase in the localization of the 60 kDa ENT1 protein in the membrane fraction of hippocampal tissue slices that were incubated with the ENT1 inhibitor NBTI, at room temperature. In addition to the changes observed in the 60 kDa protein band, the membrane localization of the 50 kDa ENT1 was significantly enhanced after treatment with NBTI (10nM), when compared to the control. A change in the protein level of the 50 kDa band was not seen with incubation of CK2 inhibitors.

In comparison to our findings, a study done by Torres et al. in 1992, on adrenal chromaffin cells, showed that NBTI binding caused quick internalization of the ENT1 transporter

protein (Torres et al., 1992). While it is possible that the inconsistencies between our study and the one by Torres and colleagues may be due to differences in the temperature that the two studies were performed at, we did not observe any significant change in the localization of the ENT proteins at 35°C, which is a more physiological temperature and closer to that used by Torres et al. However, the opposing effects of NBTI on ENT1 localization may also reflect differences in the two experimental models or the treatment incubation times.

In this set of experiments, neither concentration of NBTI caused any significant change in the membrane localization of ENT2 at room temperature. This was expected since NBTI is a highly selective inhibitor of ENT1, and therefore, should not have an effect on the ENT2 transporter at the concentrations used (Baldwin et al., 1999). However, the non-selective ENT inhibitor, DPY had no significant effect of the localization of ENT2 in the membrane fraction of the cells treated at room temperature. The extracellular adenosine tone is known to be 2-fold higher at room temperature than it is at 32°C, which is speculated to result from the reduced function of ENT2 at lower temperatures (Dunwiddie and Diao, 2000). Thus, it is possible that a reduction in the membrane localization of ENT2 would not be detectible because its membrane localization is already quite low at this temperature.

In the membrane fraction of the cell, inhibition with NBTI at room temperature did not cause a statistically significant ($p>0.05$) change in localization of GluR1 or GluR2. Although a down-regulation of these subunits was anticipated, adenosine transport blockers can have varying effects on extracellular adenosine concentrations depending on the extent to which they block either efflux or uptake of adenosine (Phillis et al., 1989; Andine et al., 1990; Gu et al., 1995; Gidday et al., 1996). Therefore, the inhibition of adenosine transport may not have been sufficient to elevate the extracellular adenosine tone to the level needed for prolonged A1 receptor activation and the subsequent reduction of GluR1/GluR2 membrane localization.

There was no significant change in the localization of any of the proteins studied at 35°C and there were no significant changes in the localization of any of the proteins in the cytosolic fraction at room temperature or 35°C. However, since there were no significant changes among the proteins studied at the higher temperature, these data suggest that enhanced protein degradation was likely a confounding factor, making it difficult to explain any CK2-mediated changes in ENTs localization at more physiological temperatures.

5.6 Future directions

As discussed previously, the CK2-mediated phosphorylation of ENTs has the potential to modify synaptic transmission in such a way that makes hippocampal neurons more vulnerable to excitotoxicity. The literature has yet to show substantial functional evidence to support this hypothesis. Consequently, further experimentation is required to confirm that an increase in extracellular adenosine concentration after CK2 inhibition alters AMPA receptor-mediated synaptic transmission and neuronal health. The work presented in this thesis suggests that ENT surface localization is influenced by CK2-mediated phosphorylation, that being said, it would be interesting to determine whether the surface localization of ENTs in ENT1 over-expressing mice (Parkinson et al., 2009) is more susceptible to manipulation of CK2. Moreover, to prove the direct phosphorylation of ENTs by CK2, site directed mutagenesis of the serine moiety in ENT1 and ENT2 and localization studies in a transient expression system in cell culture could be performed (Han et al., 1999).

CHAPTER 6

CONCLUSION

The results of this thesis provide novel evidence that inhibition of CK2 activity decreases the concentration of ENT1, ENT2 and GluR1 and GluR2-containing AMPA receptors in the plasma membrane fraction of rat hippocampal tissue. Comparison of levels in membrane and cytosol suggest this may be due to changes in localization of these proteins mediated by CK2 activity. Together, these results suggest that CK2-mediated phosphorylation can influence the localization and function of ENTs and AMPA receptor subunits. Unpublished data from our lab also showed that during hypoxia the CK2 inhibitors caused a more marked inhibition of surface localization of ENT1 and GluR2 subunits, implying that these changes would exacerbate ischemia-induced neuronal damage (personal communication, Z. Chen). This is an important finding since memory loss is one of the most common symptoms following a stroke and can be caused, in part, by neuronal damage in the hippocampus. Harnessing this pathway as a means to control extracellular levels of adenosine could provide neuroprotective targets during ischemic insult to the brain.

CHAPTER 7

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