Interactions of AMPAR-Adenosine Receptor-Equilibrative Nucleoside Transporter in the Hippocampus: Implication for Stroke

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ABSTRACT

The activation of presynaptic adenosine A1 receptors (A1Rs) is known to cause profound synaptic depression during hypoxia/cerebral ischemic insults, but postsynaptic function of A1Rs are still unclear. The goal of the current study is to provide a more comprehensive view of adenosinergic signaling. Firstly, I established that A1Rs and GluA2-containing AMPARs formed stable protein complexes from hippocampal brain homogenates and cultured hippocampal neurons. In contrast, adenosine 2A receptors (A2ARs) did not co-precipitate or colocalize with GluA2-containing AMPARs. Secondly, by using different approaches I have confirmed that prolonged stimulations of A1Rs with the agonist CPA was found to cause adenosine-induced persistent synaptic depression (APSD) in hippocampal brain slices, and APSD levels were blunted by inhibiting clathrin-mediated endocytosis of GluA2 with the Tat-GluA2-3Y peptide. This was initially demonstrated by biotinylation assays and membrane fractionation, in which prolonged CPA incubation showed a significant depletion of both GluA2 and GluA1 surface expression from hippocampal brain slices and cultured hippocampal neurons. In contrast, Tat-GluA2-3Y peptide or dynamin inhibitor Dynasore prevented CPA-induced GluA2 and GluA1 internalization. Additionally, confocal imaging analysis confirmed that functional A1Rs, but not A2ARs, are required for clathrin-mediated endocytosis of AMPARs in hippocampal neurons. Pharmacological inhibitors and shRNA knockdown of p38 MAPK and JNK were found to prevent A1R-mediated internalization of GluA2 but not GluA1 subunits. However, Tat-GluA2-3Y peptide or A1R antagonist DPCPX can prevent the hypoxia-mediated internalization of both GluA2 and GluA1. Finally, in the pial vessel disruption (PVD) cortical stroke model, reduced hippocampal GluA2, GluA1, and A1R surface expression and synaptic depression have been shown in hippocampal slices from a unilateral cortical lesioned brain compared to sham brain, which is consistent with our previous results of AMPAR downregulation and decreased probability of transmitter release. The PVD-lesioned brains also displayed increased hippocampal neurodegeneration compared to sham brains. Taken together, these results indicate a previously unknown mechanism that A1R-induced persistent synaptic depression involves clathrin-mediated GluA2 and GluA1 internalization in hypoxia/cerebral ischemia.

Both equilibrative nucleoside transporters (ENTs) and A1Rs are widely expressed in the hippocampus, and regulate extracellular adenosine level and induce synaptic depression, respectively, during cerebral ischemia. However, the cellular mechanisms that control the cell surface expression of ENTs and A1Rs in the brain remain poorly resolved. Since ENTs contain consensus sites for Casein Kinase 2 (CK2) phosphorylation, I tested the hypothesis that ENT and A1R interactions and CK2 inhibition are involved in A1R-dependent downregulation of ENT surface expression during hypoxia. Coimmunoprecipitation from rat hippocampal brain homogenates and confocal imaging microscopy of primary cultured hippocampal neurons revealed physical associations of ENTs with A1Rs, but not with A2ARs. Using whole lysates and membrane fractions from hippocampal brain slices and a phospho-specific antibody to immunoprecipitate the phosphoSerine254-ENT1 (pSer254-ENT1, a known CK2 target), I then determined that ENT1 was constitutively phosphorylated. Several CK2 inhibitors (TBB, DMAT, and DRB), but not the ENT1-selective inhibitor (NBTI) reduced pSer254-ENT1 level in whole hippocampal lysates. DRB also decreased, while CK2 activator spermine increased, the surface expression of pSer254-ENT1 in biotinylation assays of hippocampal brain slices. Moreover, biotinylation of cultured hippocampal neurons revealed that ENT1 and ENT2 surface expression was downregulated by CK2 or ENT inhibitors and by A1R agonist CPA, but not in the presence of A1R antagonist DPCPX. Pretreatments of hippocampal slices with CK2 or ENT blockers also enhanced hypoxia-mediated downregulation of ENT and A1R surface expression. These results indicate that CK2-induced and A1R-linked ENT trafficking represents an important regulatory mechanism of hypoxic/ischemic hippocampal brain damage.

The high prevalence of neurodegenerative disorders that accompany memory deficits occurs in the elderly, including stroke and Alzheimer's disease, and it is also known that extracellular levels of adenosine are enhanced in aged brains. To determine whether the mechanisms we previously identified for A1R-mediated AMPAR internalization also contribute to dysfunction in synaptic plasticity in aged brains, I compared surface levels of AMPARs from hippocampal slices of young (1 month) and old (7-12 months) animals. I found that surface expression of AMPARs decreased in aged hippocampus. To study changes in synaptic plasticity, I then performed

electrophysiological studies to compare chemically-induced long term potentiation (cLTP) in the hippocampus of young and old rats. Consistent with the biochemical results, I demonstrated that aged hippocampal slices displayed impaired cLTP, which suggests that aging impaired synaptic plasticity by promoting decreased surface expression of AMPARs. Next, I evaluated the surface levels of AMPARs before and after cLTP in young and old hippocampus to determine whether basal clathrin-mediated endocytosis of AMPARs contributes to impairments in cLTP. Following the cLTP induction, brain slices were analyzed biochemically. Under basal conditions, I showed that young brains contained higher levels of surface-expressed AMPARs compared to older brains. To test the hypothesis that this difference in baseline AMPAR surface expression contributes to cLTP deficits and was likely due to increased rate of endocytosis associated with enhanced adenosine tone in aged brains, I demonstrated with the use of two blockers of endocytosis pathways (Tat-GluA2-3Y peptide and Dynasore) that cLTP could be similarly enhanced in the young and older brains. Therefore, these results indicate that increased adenosinergic signaling in aged brains leads to increased endocytosis of AMPARs and impaired synaptic plasticity.

Together, these data suggest that interactions of AMPAR-A1R-equilibrative nucleoside transporter in the hippocampus regulate glutamatergic synaptic transmission, and enhanced A1R signaling increases both neurodegeneration in ischemic conditions and synaptic impairments in ischemic and aged brains.

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LIST OF ABBREVIATIONS

A1R	Adenosine A1 receptor
A2AR	Adenosine A2A receptor
ACSF	artificial cerebrospinal fluid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APSD	adenosine-induced persistent synaptic depression
ATP	adenosine triphosphate
BDNF	brain-derived neurotropic factor
Ca^{2+}	calcium
cAMP	cyclic-AMP
cDNA	complimentary deoxyribonucleic acid
CK1	protein kinase 1
CK2	protein kinase 2
CNS	central nervous system
CNT	concentrative nucleoside transporter

CPA	N6-cyclopentyl-adenosine
CRE	cAMP response element
CREB	cAMP response element-binding
DMAT	dimethylaminotetrabromobenzimidazole
DMSO	dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3--dipropylxanthine
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
GluA	glutamate receptor
GTP	guanosine triphosphate
JNK	c-Jun N-terminal kinase x
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
PVD	pial vessel disruption
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SAH	S-adenosylhomocysteine
shRNA	small hairpin RNA
TBB	tetrabromobenzotriazole

CHAPTER 1

1. Introduction

Stroke is one of the major causes of death and long-term injuries worldwide (Macrez, Ali et al. 2011). According to the Statistics Canada, stroke is the third top cause of death in Canada. In 2011, about 315,000 people were suffering from stroke in Canada. Six percent of all deaths in Canada (over 14,000 Canadians) resulted from stroke in 2012. As stated by statistics of the Heart and Stroke Foundation of Canada, 80% of strokes are of the ischemic type which arises as a result of the interruption of blood flow to the brain.

The hippocampus located in the temporal lobes is the memory-forming center, which is highly sensitive to hypoxic-ischemic injuries in the central nervous system (CNS) (Yue, Mehmet et al. 1997). Hypoxic-ischemic injuries could lead to neuronal cell death by both apoptosis and necrosis (Yue, Mehmet et al. 1997; Yuan and Yankner 2000). The consequence of cell death in the brain could be increased release of neurotoxic intracellular molecules (e.g., ATP, divalent cations, and cytokines), increased intracellular calcium elevation, and accumulation of glutamate in the extracellular space (White, Sullivan et al. 2000; Hertz 2008). To improve the outcome after stroke, more than 1000 pharmacologic neural protective molecules were identified and more than 250 clinical trials were implemented, however, so far none of them has completed phase III clinical trials (Young et al., 2007; Moskowitz et al., 2010; Albers et al., 2011; Macrez et al., 2011; Thauerer et al., 2012). As metabolites and precursors of nucleotides, nucleosides play an initial role in nucleic acid synthesis. The nucleoside adenosine plays a crucial role in the regulation of multiple physiological processes, including renal function and neurotransmission (Elwi, Damaraju et al. 2006). The adenosinergic signaling in stroke has been the subject of intense research over the past four decades, but the precise contribution of adenosine-related proteins (e.g., adenosine receptors, transporters, adenosine kinases, and adenosine deaminases) to synaptic transmission and neuronal death warrants further investigations. Therefore, remarkable progress in mechanisms of stroke is needed to advance the search for novel neuroprotective approaches in stroke.

1.1 Adenosine signaling

Adenosine is well accepted to be a neuromodulator metabolite and neuroprotective. Various methods have been used to measure the concentration of adenosine in brain fluid, such as the cortical cup technique (Phillis 1989) or the microdialysis (Pazzagli, Pedata et al. 1993) technique reviewed by (Pedata, Corsi et al. 2001). By microdialysis experiment, adenosine concentration was measured in the nM range under normoxic conditions and in the μM range under ischemia (Pedata, Corsi et al. 2001). During *in vitro* ischemia experiments, adenosine concentration reached 30 μM, and at this level of adenosine, all subtypes of adenosine receptors would be expected to be stimulated (Pedata, Corsi et al. 2001). Adenosine is mainly derived from ATP and *de novo* synthesis of adenosine is low in brain (Camici, Micheli et al. 2009). Intracellular adenosine is mainly from degraded ATP by adenylate kinase to ADP and AMP, then, cytosolic 5'-nucleotidase further breaks down AMP to adenosine (Pedata, Corsi et al. 2001). Another source of adenosine is the degradation of S-adenosylhomocysteine (SAH) by SAH-hydrolase; however, it is reported this pathway is not a consistent source of adenosine (Pak, Haas et al. 1994; Latini 1995). One more source of adenosine is the intracellular conversion of cyclic AMP to AMP by phosphodiesterase, and then AMP is degraded to adenosine (Craig, Temple et al. 1994).

The released ATP could be extracellularly converted to adenosine by 5'-nucleotidases (Richardson, Brown et al. 1987; Terrian, Hernandez et al. 1989). Extracellular adenosine could also be derived from extracellular cyclic AMP which is degraded by ecto-phosphodiesterases (Rosenberg and Dichter 1989; Rosenberg and Li 1995). During hypoxic/ischemic condition, adenosine comes from both intracellular (Lloyd, Lindstrom et al. 1993; Cunha, Vizi et al. 1996) and extracellular sources (Meghji, Tuttle et al. 1989).

Adenosine concentrations under normoxic physiological condition, as measured by *in vivo* cortical cup technique, are in the range 30-50 nM in the cerebral cortex (Phillis 1989). However, when measured by microdialysis fiber implantation, adenosine concentrations are estimated to be between 40-210 nM in the striatum (Ballarin, Fredholm et al. 1991; Pazzagli, Corsi et al. 1995), and 109 nM in the cortex (Pazzagli, Corsi et al.

1994), as well as 120-200 nM in hippocampus (Dunwiddie and Diao 1994). In contrast, under global ischemic conditions (Hagberg, Andersson et al. 1987; Dux, Fastbom et al. 1990), adenosine concentrations were much higher between 24-40 μM by microdialysis technique.

1.2 Adenosine receptors

Four adenosine receptors have been identified as A1, A2A, A2B, and A3 receptor subtypes. All receptors are members of a family of G-protein coupled receptors, with A1 and A3 interacting with Gi/Go proteins while A2A and A2B are coupled to Gs (Palmer and Stiles 1997). Functionally, adenosine A1 and A2A receptors have higher affinity for adenosine among all the subtypes, with A1 having a slightly higher affinity than A2A (von Lubitz 2001). A1Rs are abundant in the hippocampus, superior colliculus, cortex, and cerebellum (Fastbom, Pazos et al. 1987; Jarvis and Williams 1989). A1 receptors are abundantly distributed in both presynapses and postsynapses (Tetzlaff, Schubert et al. 1987). Adenosine receptors are also known to interact with other receptors, including dopamine D2 receptor (Agnati, Ferre et al. 2003).

A2ARs exist throughout the brain but the density is considerably lower compared to the density of A1Rs (Fredholm, Cunha et al. 2003). Because of availability of fairly selective pharmacological A1R agonists (e.g., CPA) and antagonists (e.g., DPCPX), A2AR agonists (e.g., CGS 21680) and antagonists (e.g., SCH 58261), and receptor knockout mice, the specific roles of A1Rs and A2ARs in brain function have been well documented. For example, A1Rs are generally believed to be neuroprotective and involved in synaptic depression, while A2ARs mainly mediate excitatory responses and contribute to neurotoxicity (Fredholm 1997; Dunwiddie and Masino 2001). The adenosine 2B and adenosine 3 receptors, on the other hand, are less well characterized due to their general low abundance of expression in brain tissue. Adenosine 3 receptors are present throughout the whole brain with the lowest density compared to the other subtypes (Ji, Lubitz et al. 1994).

1.3 Function of adenosine receptors

It is widely accepted that adenosine has a neuroprotective role when imbalance of energy use and delivery happens during ischemic or hypoxic conditions (Thauerer, Zur Nedden et al. 2012). Rather than adenosine directly transferring information to cells, adenosine acts via adenosine receptors to modulate the flow of information between cells (Cunha 2005). The functions of adenosine receptors include the regulation of transmitter release and nerve activity, by virtue of their functional or physical associations with other transmitter systems and calcium channels (e.g., the exocytotic machinery and N-type calcium channels in the presynaptic membranes) (Fredholm, Chen et al. 2005). A1Rs and A2ARs regulate the release of various neurotransmitters, such as glutamate and dopamine (Fuxe, Ferre et al. 2007; Cunha, Ferre et al. 2008). A1Rs inhibit presynaptic glutamate release (Proctor and Dunwiddie 1987; Thompson, Haas et al. 1992) and lead to neuronal hyperpolarization by activating postsynaptic potassium conductance (Greene and Haas 1991). In contrast, A2ARs facilitate the release of many neurotransmitters , such as glutamate and glycine in the brain (Cunha 2005). Depending on the adenosine receptor subtypes being activated and subcellular location of this activation (either pre- or post-synaptic), these adenosine receptors could bind adenosine to produce very distinct actions in the different regions of the brain (Cunha 2005). A1Rs inhibit nerve activity by a predominantly presynaptic action, while A2ARs mainly promote transmitter release (Muller 2001; Yu, Shen et al. 2008). Consequently, agonist of A1R and antagonist of A2ARs are widely believed to act as neuroprotective agents in the brain.

Although A1R and A2AR have apposite functions, previous studies have shown interactions between them in hippocampal neurons (O'Kane and Stone 1998; Ciruela, Casado et al. 2006; Ciruela, Ferre et al. 2006). Adenosine receptors can also interact with other classes of receptors. For example, A2ARs have been shown to form heteromeric complexes with dopamine D2 receptors (D2Rs) (Al-Hasani, Foster et al. 2011) or metabotropic glutamate 5 receptors (mGluR5) (Rodrigues, Alfaro et al. 2004). These heteromeric associations between adenosine receptors and other receptors have been suggested to alter the affinities of adenosine receptors to endogenous adenosine or alter the surface trafficking of other receptors. It was reported that D2Rs have increased desensitization in absence of A2ARs in ventral tegmental area (Al-Hasani, Foster et al.

2011). This may be of physiological relevance depending on the brain locations these heteromeric associations are taking place.

1.4 Adenosine receptor trafficking

Agonist-induced adenosine receptor desensitization and trafficking could occur through different mechanisms, which can include arrestins, second messenger-dependent kinase regulation, actions of different G proteins, and other non-clathrin-dependent internalization pathways (Mundell and Kelly 2011). The trafficking of adenosine receptors is important for modifying the response of adenosine under physiological and pathological conditions (Jacobson and Gao 2006; Fredholm 2010). Therefore, before discussing the effects of adenosine receptor activation on other receptor trafficking, it is necessary to provide an introduction to trafficking of adenosine receptors. A1Rs internalize in cerebral ischemia (Daval, Von Lubitz et al. 1989; Aden, Lindstrom et al. 1994; Nagasawa, Araki et al. 1994). A1Rs internalize slower (within several hours) than A3 receptors (within minutes) (Palmer and Stiles 1997) and A2ARs desensitize much faster than A1Rs (Klaasse, Ijzerman et al. 2008), suggesting that agonist binding of different adenosine receptors leads to different rates of internalization and to different levels of contribution to regulation of neuronal function under normal or pathological conditions distinct of each other.

Because adenosine receptors are G-protein-coupled receptors, the general mechanisms of G-protein-coupled receptor trafficking are firstly introduced. In general, receptor trafficking not only refers to the receptor internalization, but it also refers to the forward trafficking of receptors (i.e., insertion of receptors) to the plasma membrane, recycling and degradation of receptors. Specific to neurons, receptor trafficking could also include the movement of receptors from neuronal dendritic spines to shaft. The well-addressed mechanism of adenosine receptor trafficking involves the arrestin-dependent and clathrin-mediated internalization pathway (Ferguson, Watterson et al. 2002; Reiter and Lefkowitz 2006). The arrestins are scaffolding proteins that could initiate alternative signaling pathways by coupling different proteins like mitogen-activated protein kinases (MAPK) to surface receptors (Song, Coffa et al. 2009). Other mechanisms of G-protein-

coupled receptor trafficking involve enrichments of these receptors in domains, such as caveolae and cholesterol-enriched lipid rafts (Chini and Parenti 2004).

The topic of adenosine receptor trafficking has been studied for two decades. The A1Rs could be phosphorylated on serine and threonine sites by activation of PKA or PKC (Ciruela, Saura et al. 1997). Arrestin has been shown to decrease plasma membrane levels of A1Rs, and arrestin knockdown abolished agonist-induced MAP kinase activation, suggesting important roles of the arrestin-family of scaffolding proteins in regulating A1R trafficking and coupling to downstream intracellular signaling cascades (Jajoo, Mukherjea et al. 2010).

1.5 Adenosine receptor and stroke

The neuroprotective role of adenosine via adenosine receptors has been suggested in neurodegenerative diseases, such as stroke, epilepsy, Parkinson's and Alzheimer's disease (Stone, Ceruti et al. 2009; Gomes, Kaster et al. 2011). Adenosine normally acts as a neuroprotective modulator by activating A1Rs and inhibiting neurotransmitter release, but it can also act as an excitatory agent by acting on A2ARs (Cunha 2005).

During stroke, activation of A1R is relevant to the inhibition of glutamate release which results from A1R-mediated inhibition of presynaptic calcium currents (Fredholm and Dunwiddie 1988; Prince and Stevens 1992). The selective A1R agonist, CPA, also has huge inhibitory effects on glutamate release (Cascalheira and Sebastiao 1998), whereas stimulation of A2ARs by agonist CGS 21680 has excitatory effects (O'Regan, Simpson et al. 1992; Popoli, Betto et al. 1995). Agonists of A1R are neuroprotective, whereas antagonists of A1R enhance damage to neurons (De Mendonca, Sebastiao et al. 2000). However, paradoxically chronic A1R activation could initiate and even enhance brain damage in animal models of stroke (Jacobson, Von Lubitz et al. 1996). In contrast, inhibition of A2ARs decreases neuronal damage in neurodegenerative diseases (Phillis 2000; Brambilla, Cottini et al. 2003).

1.6 AMPAR subunits and AMPAR compositions

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (also known as AMPA receptors or AMPARs) are a subclass of ionotropic glutamate receptors found in virtually all excitatory synapses, and are comprised of multimeric protein assemblies likely consisting of combinations of four subunits termed GluA1-4 or GluAA-D (Wisden and Seeburg 1993; Malenka 2003). All subunits include an extracellular domain, a cytoplasmic C-terminal tail, three transmembrane domains and a membrane re-entrant hairpin that forms the pore loop (Tichelaar, Safferling et al. 2004). The re-entrant hairpin loop contains the Q/R editing site. GluA1 and GluA4 have long C-terminal domains and GluA2 and GluA3 contain shorter C-terminal domains (Anggono and Huganir 2012). The C-termini of AMPAR subunits contain the regulatory sites for protein phosphorylation, as well as binding sites for interacting with numerous signaling proteins that are important for AMPAR trafficking and function (Anggono and Huganir 2012). In the mature hippocampus, two types of AMPARs appear to dominate: heteromers consisting of GluA1/2; or GluA2/3 (Wenthold, Petralia et al. 1996). Synaptic AMPARs are mainly represented by the heteromeric combinations of GluA1 and GluA2 (Lu, Shi et al. 2009).

1.7 AMPAR assembly

Assembly of AMPARs happens in the endoplasmic reticulum (ER) (Ayalon and Stern-Bach 2001). AMPAR subunits form as dimers, then dimer-of-dimers assemble as a tetramer (Tichelaar, Safferling et al. 2004). RNA editing determines AMPAR assembly at the step of tetramerization, particularly, in GluA2 (Seeburg 1996). In the RNA Q/R editing site in the hairpin re-entrant loop region, an arginine residue (R607) is replaced by the glutamine residue Q607 in GluA1, GluA3 and GluA4 (Greger, Khatri et al. 2003). Edited R subunits are mainly ER retained and unassembled, whereas unedited Q subunits mature, tetramerize and traffic to synapses (Greger, Khatri et al. 2003), where they form functional and calcium-permeable homomeric AMPARs. Unedited Q-subunits tetramerize and move to cell surface, whereas the edited R-subunits are still dimeric and ER-retained unless coupled with unedited Q-subunits (Greger, Khatri et al. 2002). Taken

together, the single amino acid in the hairpin loop can regulate the assembly, trafficking and function of AMPARs.

1.8 Phosphorylation of AMPARs

For regulation of AMPARs, the phosphorylation modulation is also important. AMPARs trafficking and memory formation requires CaMKII (Malinow, Schulman et al. 1989). Phosphorylation of GluA1 by CaMKII at S831 (Roche, O'Brien et al. 1996; Barria, Derkach et al. 1997; Mammen, Kameyama et al. 1997), PKA at S845 (Roche, O'Brien et al. 1996), and PKC at S818 and S831 (Roche, O'Brien et al. 1996; Boehm, Kang et al. 2006), all enhance AMPAR trafficking to synapses. Phosphorylation of GluA1 at S831 could enhance the conductance of GluA1 homomers and GluA1/2 heteromers (Kristensen, Jenkins et al. 2011). CaMKII phosphorylates the AMPAR-interacting protein stargazin, which is an auxiliary AMPAR subunit and helps anchor AMPAR at synaptic sites (Jackson and Nicoll 2011). The PKC family is crucial for expression of LTP and memory formation (Colley, Sheu et al. 1990). Inhibition of PKC blocks LTP expression (Colley, Sheu et al. 1990), whereas activation of PKC rescues NMDA receptor blocked LTP (Kleschevnikov and Routtenberg 2001). Phosphorylation of GluA2 at S880 (Chung, Xia et al. 2000; Seidenman, Steinberg et al. 2003) and dephosphorylation of GluA1 at S845 and S831 (Kameyama, Lee et al. 1998; Lee, Barbarosie et al. 2000) decrease the surface expression of GluA1 which leads to synaptic weakening mimicking a long term depression (LTD)-like process.

1.9 AMPAR-interacting proteins

A wide range of AMPAR-interacting proteins is known to regulate AMPAR trafficking. A majority of these proteins binds to the carboxyl terminus of the GluA2/3 AMPAR subunits, and they include the N-ethylmaleimide-sensitive factor (NSF) and the PDZ-domain-containing proteins GRIP1 (glutamate-receptor-interacting protein), GRIP2/ABP and PICK (protein that interacts with C kinase) (Braithwaite, Meyer et al. 2000; Scannevin and Huganir 2000). C-termini of GluA1, 2, 3 bind to scaffolding proteins that contain a single or several PDZ domains (Kim and Sheng 2004). PDZ-containing

proteins have an 80-90 amino acid PDZ domain, which binds and stabilizes transmembrane proteins, including AMPARs (Kim and Sheng 2004). PDZ is an acronym of the first letter of three of the first proteins discovered to contain this domain, which are PSD-95, disc large tumor suppressor (Dlg1), and zona occludens-1 (zo-1) (Kim and Sheng 2004). Other examples of proteins that interact with AMPARs via direct interaction with PDZ domains include SAP97 (synaptic associated protein 97 kDa) and NARP (neuronal activity-regulated pentraxin). SAP97 binds to the last three distal amino acids (TGL) of the carboxyl terminus of GluA1 via a PDZ domain, while NARP binds to the extracellular amino terminus of GluA1, 2 and 3 (O'Brien 1999). PDZ-domain-containing proteins that bind to the carboxyl terminus of GluA2/3 regulate AMPAR transport, and include the scaffolding proteins GRIP1, a protein that contains seven PDZ domains, ABP and GRIP2, which are splice variants with PDZ domains, and the PKC-interacting protein PICK1, which has one PDZ domain (Carroll, Beattie et al. 2001). GluA1 binds to SAP97 (Leonard, Davare et al. 1998). SAP97 is a member of the membrane-associated guanylate kinase (MAGUK) proteins (Montgomery, Zamorano et al. 2004). Phosphorylation of SAP97 by CaMKII regulates the synaptic trafficking of GluA1 containing AMPARs (Mauceri, Cattabeni et al. 2004).

N-ethylmaleimide-sensitive factor (NSF)

GluA2 directly associates with NSF and this interaction is Ca^{2+}-dependent (Hanley 2007). NSF interacts with the intracellular tail of GluA2 and that disruption of this interaction causes a decrease in synaptic efficacy that was subsequently shown to be due to the loss of synaptic AMPARs (Osten 1998). NSF binding prevents interaction between GluA2 and AP2 (the endocytic adaptor protein), and also blocks GluA2/PICK1 interaction to inhibit AMPAR internalization (Hanley, Khatri et al. 2002; Lee, Liu et al. 2002). Additionally, normal synaptic expression of AMPARs might require the NSF-GluA2 interaction (Luthi 1999). A Tat-peptide used in my studies targets the clathrin-mediated internalization of GluA2 subunits by preventing the binding of the AP2 endocytic protein (Ahmadian, Ju et al. 2004).

1.10 AMPAR trafficking

The number of AMPAR on the surface at synapses is crucial and dependent on the rates of endocytosis, exocytosis, biosynthesis and degradation. Increased exocytosis and recycling appears in LTP, while enhanced endocytosis occurs in LTD (Shepherd and Huganir 2007; Kessels and Malinow 2009; Anggono and Huganir 2012). Activation of NMDA receptors caused clathrin-mediated endocytosis of AMPARs (Lu, Helton et al. 2007). AMPARs traffic in and out of synapses through multiple pathways (Groc and Choquet 2006). AMPAR trafficking could also alter neuronal dendritic spine morphogenesis (Hanley 2008).

By directly visualizing a fusion protein of GluA1-GFP, it has been shown that LTP caused the delivery of the GluA1-GFP homomers to the synaptic plasma membrane, as inferred from electrophysiological measurements showing that increased insertion of homomeric GluA1 receptors led to the expression of a more robust inward rectification of the current-voltage relationship (a well-known biophysical fingerprint of GluA2-deficient AMPARs), whereas the endogenous GluA2-containing AMPARs did not (Shi, Hayashi et al. 1999). Moreover, GluA1-GFP homomers were not delivered constitutively to synapses but required activity-dependent trafficking (Malenka and Nicoll 1999). Surprisingly, mutagenesis of the carboxy-terminal tail of GluA1 revealed that phosphorylation of serine 831, a known CaMKII site, was not required for the regulated synaptic delivery of GluA1. However, the PDZ-binding domain at the carboxyl terminus of GluA1 was needed (Barria, Derkach et al. 1997). This result means that PDZ-domain-containing proteins that interact with GluA1 plays an important role in synaptic plasticity, such as SAP97 (Leonard, Davare et al. 1998). Interestingly, CaMKII or LTP is not necessary for GluA2-containing AMPARs being delivered to the synapse. Furthermore, the delivery of AMPARs to synapses during early postnatal development seems to require GluA4 subunits and to use an activity-dependent mechanism distinct from that used during LTP (Zhu, Esteban et al. 2000). These studies indicate that the roles of specific AMPAR subunits in regulated endocytosis and activity-dependent exocytosis/delivery might differ, presumably because of the difference in their specific protein-protein interactions.

Stargazin interacts with both AMPAR subunits and synaptic PDZ proteins, such as PSD-95, which seems to be necessary for the synaptic clustering of the AMPARs,

suggesting that this or other AMPAR-interacting proteins might be involved in the regulated or constitutive exocytosis of AMPARs (Chen 2000). Greater levels of GluA1 and GluA2 in the synaptic membrane fractions have also been found in NMDA-potentiated slices (Broutman and Baudry 2001). This elevation of synaptic AMPARs required the activity of CaMKII and the calcium-dependent protease calpain, which was blocked by brefeldin A, an inhibitor of protein transport from the ER to Golgi apparatus. This raises the possibility that AMPAR-containing vesicles are being secreted from the Golgi during regulated receptor insertion, suggesting that constitutively recycling receptors and actively inserted receptors are not, at least initially, in the same pool (Broutman and Baudry 2001).

Electrophysiological, biochemical and immunocytochemical techniques are used to study the regulation of trafficking of AMPARs in cultured neurons following activation of synaptic NMDARs. An increase in synaptic AMPAR mediated currents has been demonstrated with this treatment that mimics an increase in activity-dependent process (Lu 2001). There is more evidence for the activity-dependent synaptic delivery of endogenous AMPARS comes from the findings that after the induction of LTP *in vivo* in the adult hippocampus, there is an increase in the amount of GluA1 and GluA2 in synaptoneurosomes (Heynen, Quinlan et al. 2000).

A review of the functions and mechanisms of AMPAR endocytosis would not be complete without mentioning the converse process of AMPAR exocytosis. Constitutive AMPAR insertion has also been detected immunocytochemically in hippocampal neurons and biochemically in cortical neurons, and the estimated rate of reinsertion of internalized receptors is tightly coupled to the rate of endocytosis, resulting in a relatively constant level of surface AMPAR expression (Ehlers 2000). Inhibition of exocytosis results in a run-down of synaptic responses; however, blockade of clathrin-mediated endocytosis in hippocampal neurons results in a rapid increase in synaptic currents (Luscher 1999). Using biochemical analysis, AMPARs have also been shown to recycle back to the membrane surface after the triggering of regulated receptor endocytosis (Ehlers 2000). It is now widely known that PKA-, PKC-, and CaMKII-mediated phosphorylation of GluA1 subunits could be important for AMPAR reinsertion (Ehlers 2000; Diering, Gustina et al. 2014).

1.11 Regulation of AMPAR trafficking

The relationship between synaptic strength and AMPAR trafficking is dependent on the composition of AMPAR subunits. Firstly, long-tailed (like GluA1) subunit-containing AMPARs moving to synapses cause synaptic strengthening. Secondly, activity-dependent endocytosis of short-tailed or long tailed AMPARs from synapses leads to synaptic weakening, while short-tailed AMPAR constitutive migration to synapses cannot change synaptic strength (Kessels and Malinow 2009). AMPAR trafficking to synapses after stimuli has also been shown to involve subunit switching, with the GluA1/2 subunits being replaced by GluA2/4 subunits, and this subunit switching has been proposed as a mechanism to stabilize the strength of synapses (Kessels and Malinow 2009).

Long-tailed AMPARs regulate the activity-driven synaptic incorporation of AMPARs (Harms, Tovar et al. 2005). In addition, heteromeric recombinant GluA1 and GluA2 are driven into synapses by LTP (Shi, Hayashi et al. 2001). Moreover, the AMPAR-interacting proteins have been shown to be important for the regulation of AMPAR trafficking.

The full-length GluA2 C-terminal tail (Shi, Hayashi et al. 2001) or a fragment of C-terminal tail blocks LTD and decreases synaptic transmission. A Tat-GluA2-3Y peptide, which only contains nine amino acids of the GluA2 C-terminal tail, abolishes activation of NMDAR-induced endocytosis of AMPAR, without changing LTP (Ahmadian, Ju et al. 2004). The Tat-GluA2-3Y peptide serves as an inhibitor of clathrin-mediated GluA2 receptor endocytosis (Brebner, Wong et al. 2005; Xiong, Kojic et al. 2006), and I subsequently used this peptide in my studies to determine the role of A1Rs in synaptic depression and AMPAR internalization.

Different methods have been used to study the internalization of AMPARs. Recombinant, tagged receptor with green fluorescent protein (GFP) and confocal microscopy have been used extensively (Passafaro, Piech et al. 2001; Sheng and Lee 2001). In addition, ecliptic pHluorin-tagged GluA2 was used to visualize changes in AMPAR surface expression in real time (Ashby, De La Rue et al. 2004).

AMPAR internalization pathways include the clathrin-mediated endocytosis protein machineries. Previous reports implicated the involvement of AMPAR endocytosis in

several forms of LTD suggested that similar intracellular signaling pathways mediate AMPAR function, transport and plasticity (Carroll, Beattie et al. 2001). Studies of AMPAR endocytosis following NMDAR activation led to the predominant hypothesis that triggering NMDAR-dependent LTD requires an NMDAR-dependent rise in postsynaptic calcium that preferentially activates a protein-phosphatase cascade that includes calcineurin (PP2B), a Ca^{2+} independent phosphatase, and protein phosphatase 1 (PP1) (Lisman 1989; Mulkey, Herron et al. 1993; O'Dell and Kandel 1994). Calcineurin was also found to be involved in promoting AMPAR endocytosis in response to application of insulin or AMPA, suggesting that this signaling pathway might be widely involved in the internalization of AMPARs (Beattie 2000; Lin 2000; Carroll, Beattie et al. 2001). However, how calcineurin exactly affects AMPAR endocytosis is unknown. Additionally, it is found that inhibition of PP1 blocks the internalization of AMPARs elicited by NMDA application (Ehlers 2000). As these studies used different methods, cells and models to detect internalized AMPARs, it is clear that the cellular mechanisms of AMPAR endocytosis may vary depending on the nature of triggering stimuli or cellular context. The triggering of plasticity of AMPARs has also been linked to activation of protein kinase C (PKC) rather than NMDAR-dependent LTD in the hippocampus (Linden and Connor 1991; Hartell 1994). These studies showed evidence that PKC-mediated phosphorylation of GluA2 and the consequent disruption of GluA2 interactions with PDZ-domain-containing proteins led to the internalization of AMPARs during long-term depression at parallel-fibre–Purkinje-cell synapses (Matsuda, Mikawa et al. 1999; Xia, Chung et al. 2000).

Thus the regulation of AMPAR endocytosis may differ depending on the location of neurons in the brain, perhaps because the subunit compositions of AMPARs differ, and consequently so would the receptor-associated proteins that might differentially regulate endocytosis (Carroll, Beattie et al. 2001). Two additional forms of AMPAR endocytosis have been identified that are indirectly involved in the expression of synaptic plasticity. It has been shown that the endocytosis of AMPARs could occur via a process involving dynamin and clathrin-coated pits, and that calcium-dependent activation of protein phosphatases leading to constitutive endocytosis of AMPARs has been observed electrophysiologically, immunocytochemically and biochemically (Luscher 1999; Ehlers

2000; Lin 2000). The rate of endocytosis process is rapid, resulting in an apparent turnover of 40-50% of surface receptors in tens of minutes (Ehlers 2000).

By studies of the transport of mutant AMPARs in a heterologous expression system, constitutive and activation-independent endocytosis of AMPARs is further distinguished from regulated endocytosis (Carroll, Beattie et al. 2001). There is also evidence in HEK293 cells indicating that independent mechanisms regulate two forms of AMPAR endocytosis. Deletion of a membrane-proximal segment of the GluA2 carboxyl terminus disrupted constitutive endocytosis but not a form of regulated endocytosis triggered by insulin. In contrast, mutations of the last 15 amino acids of the carboxyl terminus, which includes the PDZ-binding domain, had the opposite effect (Lin 2000). Some of the key players in endocytosis of AMPARs are discussed above, such as the PDZ-domain containing proteins including GRIP, ABP and PICK.

Endocytosis of AMPAR on the one hand and their insertion by exocytosis on the other hand, appear to be coupled, but the signaling cascades are not well known. For example, the molecular mechanisms which act on GluA2 at the level of early endosomes to allow for stimulus-dependent sorting to divergent pathways remains obscure, and neuron-specific proteins like Arc/Arg3.1 involved in endocytosis or NEEP21 involved in sorting of AMPARs in early endosomes, are promising candidates to biochemically link AMPAR to the general trafficking machinery (Hirling 2009).

In conclusion, our current knowledge of AMPAR endocytosis is still incompletely understood, and in particular, the precise molecular mechanisms of adenosine-mediated AMPAR trafficking which involves receptor trafficking during receptor endocytosis and receptor exocytosis still needs further investigation. In my studies, I have used more advanced imaging and biochemical studies to glean more information about AMPAR trafficking during adenosine receptor activation.

1.12 AMPAR and neuronal plasticity studies involving LTP/LTD

In an adult human brain, there are over 100 billion neurons and each neuron interacts with other neurons via thousands of synapses; thus, identifying how the synapses work that cause the behavioural changes in animal models is a major aim of

neurobiological research (Kessels and Malinow 2009). In the 1940s, Donald Hebb proposed that the persistent stimulation could strengthen the neuronal communication among a group of interconnected neurons, which led to the widely held view "Neurons that fire together, wire together." This is widely accepted as a means of encoding and storing memory (Henley and Wilkinson 2013). The first experimental proof of Hebbian plasticity was shown in rabbit hippocampal neurons; a long lasting stimulation of presynaptic cells increased postsynaptic responses (Bliss and Lomo 1973). This Hebbian postulate has inspired thousands of studies in learning and memory.

In general, high-frequency stimulation (e.g., 100Hz, 1s) could potentiate synaptic plasticity, causing long-term potentiation (LTP), whereas lower-frequency stimulation (e.g., 1 Hz, 15 min) can depress synaptic activity, leading to long-term depression (LTD) (Henley and Wilkinson 2013). LTP and LTD are believed to be cellular substrates for learning and memory (Bliss and Collingridge 1993).

Many factors are confirmed to regulate synaptic plasticity. The number and properties of postsynaptic receptors are crucial for synaptic plasticity (e.g., AMPAR contribution to LTP). LTP has two distinct phases: induction phase and maintenance phase. The induction phase of LTP is to initiate learning, which relies on post-translational modifications or rapid trafficking of AMPAR proteins (Abel and Lattal 2001). However, the maintenance phase of LTP needs de novo protein synthesis (Reymann and Frey 2007).

NMDA receptors and AMPARs are widely studied for synaptic plasticity (Morris, Anderson et al. 1986; Dudek and Bear 1992). NMDA receptors are highly permeable to Ca^{2+} and blocked by Mg^{2+} (Nowak, Bregestovski et al. 1984). Different calcium dynamics and NMDA receptor activities lead to LTP or LTD. Stimulation of synaptic NMDA receptor with a co-agonist invokes LTP (Lu 2001). A train of electrical pulses with high stimulus frequency is applied to neurons to induce a rapid and substantial Ca^{2+} influx for initiating LTP (Ismailov, Kalikulov et al. 2004). In contrast, a low-frequency stimulation evokes lower Ca^{2+} influx that leads to LTD (Yang, Tang et al. 1999). These patterns of LTP and LTD have been suggested to be the physiological correlates of learning and memory processes (Larson and Lynch 1986).

About three decades ago, it was proposed that LTP was due to an increased number of synaptic AMPARs and NMDARs (Lynch and Baudry 1984; Salter 2003; Liu,

Wong et al. 2004). However, previous studies have confirmed a prominent role of AMPAR trafficking in synaptic plasticity. GluA1-knockout mice were not able to generate LTP, suggesting GluA1 is crucial for LTP (Zamanillo, Sprengel et al. 1999). Several studies also showed that GluA2 are cycling in and out of the synapses (Ehlers 2000; Shi, Hayashi et al. 2001; Zhou, Xiao et al. 2001). Another report also indicated that an important residue, arginine substituted to glutamine (R586Q) in GluA2, can affect the surface expression of GluA2 in cultured neurons, suggesting that this arginine residue in the C-terminus is crucial for GluA2 trafficking. The AMPAR subunits with long C-terminal tails (GluA1 or GluA4) were driven by LTP or spontaneous activity into synapses, while GluA2/GluA2 homomeric AMPARs maintain constant transmission (Malinow 2003). AMPAR trafficking has also been shown to be driven by experience-dependent plasticity (Takahashi, Svoboda et al. 2003).

NMDAR-dependent LTP and LTD have been predominantly studied at synapses on pyramidal cells in the CA1 region of the hippocampus. These studies presented numerous experimental evidence for both pre- and postsynaptic modifications contributing to LTP and LTD after NMDAR stimulation (Kullmann and Siegelbaum 1995; Malenka and Nicoll 1999). Only functional NMDARs exist at synapses. The so-called 'silent synapses', under conditions of normal synaptic transmission, are so-called "silent", since these synapses are unable to respond to synaptically released glutamate owing to the voltage-dependent block of NMDARs by Mg^{2+} or they lack functional AMPARs (Malenka and Nicoll 1997). It is widely accepted now that the activity-dependent insertion of AMPARs into silent synapses could be an important mechanism for the expression of LTP. In contrast, the loss of AMPARs from AMPAR- and NMDAR-expressing synapses and the subsequent generation of silent synapses might be involved in the production of LTD (Carroll, Beattie et al. 2001).

Endocytosis of AMPARs plays a critical role in LTD. Firstly, generation of LTD in cultured hippocampal neurons decreases the number of synaptic AMPARs (Carroll, Lissin et al. 1999). Secondly, injection of the NSF inhibitory peptide causes the loss of surface AMPARs and prevents the subsequent expression of LTD (Luthi 1999). Thirdly, loading the cell with reagents that inhibit dynamin-dependent endocytosis blocks hippocampal LTD (Luscher 1999). LTD at other synaptic connections also appears to involve

endocytosis of AMPARs, such as at parallel fiber-Purkinje cell synapses in the cerebellum (Wang and Linden 2000) and excitatory synapses on dopamine cells in the ventral tegmental area (Gutlerner, Penick et al. 2002). These and other studies discussed above suggest that that AMPAR trafficking may be a universal mechanism or may be somewhat different depending on the types of protein-protein interactions involved in anchoring AMPARs to cell surface in different brain regions.

1.13 AMPARs and aging

AMPARs are known to be reduced in aging brains and in neurodegenerative diseases, such as Alzheimer's disease (Mishizen, Ikonomovic et al. 2001). Age-related loss of GluA2/3-containing AMPARs has been found in human brain (Ikonomovic, Nocera et al. 2000). In rat brain, GluA2 subunits have been found to decrease with the age of animals (Pellegrini-Giampietro, Bennett et al. 1992). Therefore, a higher stimulation is required to induce LTP (Barnes, Rao et al. 2000), partialy because of decreased AMPAR expression in aged brains, which is consistent with the observation of a recreased level of LTP detected in aged brains (Tombaugh, Rowe et al. 2002; Dieguez Jr and Barea-Rodriguez 2004). Induction of LTD has been shown to be greatly enhanced in aged brain (Norris, Korol et al. 1996). Taken together, synaptic plasticity becomes deficient during aging. Defects in trafficking of AMPAR cause deficits in synaptic plasticity during aging (Henley and Wilkinson 2013), however, more studies are warranted to determine the precise molecular links between aging and deficits in synaptic plasticity.

Fundamentally, synaptic abnormalities occur in age-related neurodegenerative disorders (Henley and Wilkinson 2013). Normal age-related cognitive decline is widely believed as an unavoidable consequence of aging (Henley and Wilkinson 2013). AMPAR dysfunction was found in multiple neurological diseases, such as Alzheimer's disease (AD), stroke and Parkinson diseases (Mishizen, Ikonomovic et al. 2001; Smith 2013). For instance, in aged brains, amyloid beta (Aβ) induced AMPAR internalization (Hsieh, Boehm et al. 2006), and CaMKII appears to be involved in this process (Gu, Liu et al. 2009).

More research is needed about the trafficking and behavior of AMPARs under normal and pathophysiological situations (Henley and Wilkinson 2013). Particularly, the molecular pathways of AMPAR trafficking and associated deficits in synaptic plasticity that accompany aging and ischemic strokes need to be further defined.

1.14 Nucleoside transporters

Transport of nucleoside is very important for nucleic acid synthesis, cytotoxicity, neurotransmission, drug transport and other processes in many kinds of cells, especially in neuron. Two main families of nucleoside transport have been well identified in mammalian cells: equilibrative nucleoside transporter (ENT, also known in humans as SLC29) and concentrative nucleoside transporter (CNT, also known in humans as SLC28). ENTs are the equilibrative, bi-directional facilitators; and CNTs are the concentrative, unidirectional Na^+/nucleoside co-transporters (Elwi, Damaraju et al. 2006; Young, Yao et al. 2008).

The human ENT family of integral membrane proteins is represented by four human protein isoforms: hENT1, hENT2, hENT3 and hENT4 (Baldwin, Yao et al. 2005). ENTs modulate cardiovascular activity and neurotransmission by regulating the concentration of adenosine available to cell surface receptors (Young, Yao et al. 2008). ENTs can facilitate the transport of both nucleosides and nucleobases through membranes. Inhibition of the function of these transporters has been suggested to increase the concentration of extracellular nucleosides, such as adenosine (Latini and Pedata 2001).

1.15 Structure of equilibrative nucleoside transporters

Knowledge of the structure of protein is very important to the understanding of the function of the protein, and provides the basis for targeting the regions known to be important for the regulation of the protein function. There are 11 transmembrane (TM) segments for hENT1, and the C-terminus is extracellular while the N-terminus is cytoplasmic (Sundaram, Yao et al. 2001). The hENT1 is N-glycosylated at a single site (hENT2 at two sites) in the large extracellular loop linking TM1 and 2, but glycosylation is not important for transport activity of hENT1 or hENT2 (Osato, Huang et al. 2003). The

TM1–6 region appears to be associated with the ability of ENT2 to transport 3´-deoxynucleosides more efficiently (Yao, Ng et al. 2001), while the TM5-6 region has been recognized in the ability of this transporter to recognise nucleobases (Yao, Ng et al. 2002).

Mutation technique is used for detecting the functional sites of the ENT sequence. For example, mutation of Gly179 in TM5 of hENT1 to Ala inhibits transport activity, and this residue has been recognized to play a direct role in NBMPR binding (SenGupta, Lum et al. 2002). However, the phosphorylation sites and their potential roles in the physiological regulation of ENT transport activity in neuronal tissue, is not yet very well characterized, and, therefore, I studied the potential role of one important protein kinase, termed casein kinase II (or CK2) in regulating ENT function as described in Chapter 3.

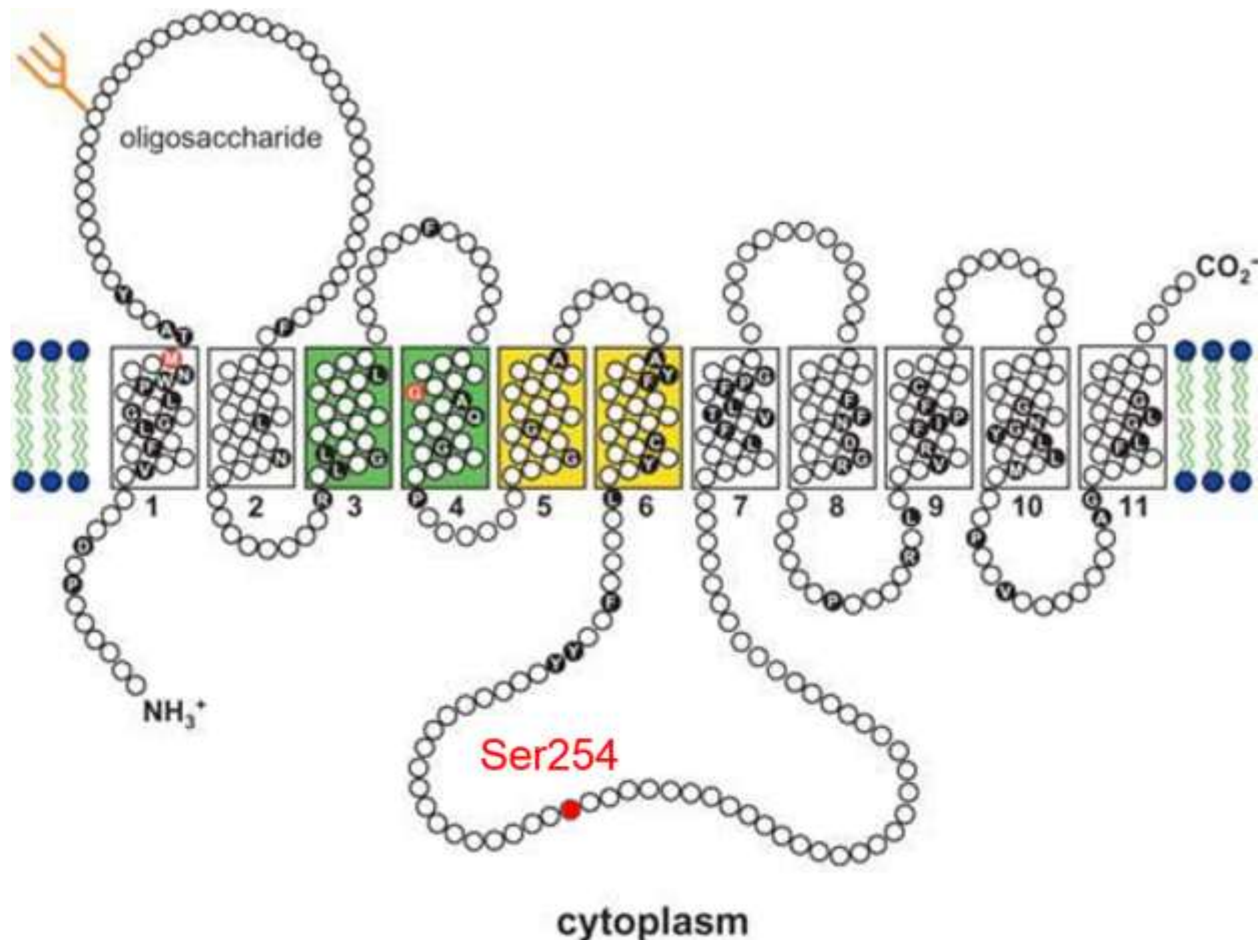

Figure 1.1 Topographical model of hENT1. This model is based on the results of glycosylation scanning mutagenesis studies and other approaches detailed in (Sundaram,

Yao et al. 2001). Predicted membrane-spanning alpha helices are numbered and the site of N-glycosylation indicated. Coloured boxes indicate the regions implicated from chimaera studies in the recognition of nucleobase substrates (yellow) and coronary vasodilator drugs and NBMPR (yellow and green). Ser254 corresponds to the CK2 consensus sequence for CK2 phosphorylation. Adapted from the reference (Baldwin, Beal et al. 2004).

ENT1

As a 456-amino acid protein, human ENT1 (hENT1) is 78% identical in sequence to the 457-amino acid-containing rat homologue (rENT1) and 79% identical to the 460-amino acid mouse protein (mENT1.1) (Yao, Ng et al. 1997). ENT1 is ubiquitously well distributed at both mRNA and protein levels. The regulation of ENT1 (and ENT2) is discussed in more detail below (in section 1.16).

ENT2

As a 456-amino acid protein, human ENT2 (hENT2) is 46% identical in amino acid sequence to hENT1 and 88% identical to the 456-amino acid mouse (mENT2) and rat (rENT2) homologues (Yao, Ng et al. 1997). ENT2 mRNA is expressed in many tissues including brain, heart, placenta, thymus, pancreas, prostate and kidney, but is particularly abundant in skeletal muscle (Griffiths, Yao et al. 1997). Human and rat ENT2 (h/rENT2) can transport a broad range of purine and pyrimidine nucleosides (Ward, Sherali et al. 2000). However, rENT2 cannot transport cytosine (Yao, Ng et al. 2002). Transport activity of h/rENT2 is lower than that of h/rENT1 and it could be inhibited by NBMPR, dipyridamole or dilazep (Crawford, Patel et al. 1998). As the surface expression of ENT2 increases, at physiological concentrations the efficiencies of nucleoside and nucleobase transport are similar for both ENT1 and ENT2 (Yao et al. 2002). As the best characterised members of the SLC29 family, both ENT1 and ENT2 are known to be facilitated diffusion transports systems (Griffiths, Yao et al. 1997). hENT2 might be included in an important pathway for cellular uptake of clinically important drugs, some of which are used in human immunodeficiency virus (HIV) therapy (Baldwin, Beal et al. 2004).

ENT3

As a 475-amino acid protein, human ENT3 (hENT3) is 29% identical in sequence to hENT1 and 74% identical to its 475-amino acid mouse homologue mENT3 (Hyde, Cass et al. 2001). The structure of hENT3 is different from hENT1 and hENT2, possessing a very long (51 residues), hydrophilic N-terminal region preceding TM1 (Baldwin, Beal et al. 2004). This special region is similar to those that mediate the sorting of other membrane proteins at the trans-Golgi network, endosomes and plasma membrane (Sandoval, Martinez-Arca et al. 2000). This difference demonstrated that these isoforms reside predominantly in an intracellular compartment, such as mitochondria, endoplasmic reticulum, or Golgi network, rather than at the cell surface (Baldwin, Beal et al. 2004), but more studies are needed to confirm this. hENT3 is widely expressed in human tissues but is particularly abundant in placenta, from which the cDNA of hENT3 was originally cloned (Hyde, Cass et al. 2001). hENT3 also has similar broad selective permeability for nucleosides and nucleobases, and has been shown to be functional in intracellular membranes (Young, Yao et al. 2008). Therefore, these differences might mean that the function of hENT3 is different from hENT1 and hENT2.

ENT4

As a 530-amino acid protein, human ENT4 (hENT4) is 86% identical in sequence to its 528-amino acid mouse homologue (mENT4) (Baldwin, Beal et al. 2004). hENT4 protein has selectivity for adenosine. Both hENT3 and hENT4 are pH-sensitive, with highest activity under acidic conditions (Young, Yao et al. 2008). This also suggests that both ENT3 and ENT4 may mainly function as cytosolic nucleoside transporters, as opposed to ENT1 and ENT2 which are most likely localized to plasma membranes whose extracellular regions are exposed to more neutral pH.

1.16 Regulation of equilibrative nucleoside transporters

The information about the mechanism of regulation of ENTs is not clear. It is reported that the rapid activation of cell surface hENT1 in non-neuronal cultured cells and cell lines has been associated with the activation of PKC by phorbol ester treatment (Coe, Zhang et al. 2002). PKC might be involved in ENT regulation, but it is not yet clear whether

changes in the phosphorylation state of the transporter itself are involved (Coe, Zhang et al. 2002). Other protein kinases have been suggested to be involved in regulating ENTs, but whether this regulation is occurring in neuronal tissue has not yet been demonstrated. Inhibition of CK2 activity or deletion of Ser254 (CK2 consensus site) from mENT1, reduces the number of functional ENT1 proteins (Bone, Robillard et al. 2007). Therefore, I proposed to further study the regulation of ENTs by CK2, in order to find some molecular links between the control of adenosine tone with levels of adenosine receptors, ENTs, and AMPARs.

The main function of ENTs is to transport nucleoside and nucleobase for salvage pathways for nucleotide synthesis. Because ENTs are also responsible for the cellular uptake of nucleoside analogues, many of which represent nucleobases for anti-cancer therapy, the transport function of ENTs is therefore now recognized as a viable way to introduce drug targets for certain diseases, including cancers (Young, Yao et al. 2008; Marechal, Mackey et al. 2009; Spratlin and Mackey 2010).

The coronary vasodilator drugs, such as dilazep, dipyridamole, lidoflazine analogues and NBMPR, as well as STI-571 (a Bcr-Abl tyrosine kinase inhibitor used in chronic myelogenous leukaemia) have been reported to be moderately potent inhibitors of ENT1 and/or ENT2 (Huang, Wang et al. 2003). However, none of these drugs, with STI-571 as exception, had been tested for possible inhibition of protein kinases. It is possible that protein kinase CK2 inhibition by these drugs may affect transport activity or surface expression of ENTs, but this needs to be further investigated. Here, in Chapter 3, I address the potential effects of known CK2 inhibitors as well as known ENT inhibitors, such as dipyridamole and nitrobenzylthioinosine (NBTI), in regulating the phosphorylation levels of ENT1 (at Ser254) and surface expression levels of ENT1 and ENT2.

As ENT inhibitors have the potential ability to raise the levels of extracellular adenosine concentrations, ENT inhibitors therefore have potential therapeutic effect in various pathologies, including cancers and stroke. The cardiovascular effect of adenosine could be enhanced and prolonged by coronary vasodilator draflazine (Dennis, Raatikainen et al. 1996). This drug effect has been attributed to ENT inhibition, which is expected to raise extracellular adenosine that underlies the beneficial effect of this drug in ischaemic diseases (Ferraro, Sardo et al. 2002).

Inhibition of the ENT1 by NBMPR leads to presynaptic A1 receptor-mediated inhibition of glutamatergic synaptic transmission (Ackley, Governo et al. 2003). In integrated brain preparations, the inhibition or blockade of equilibrative nucleoside transporters causes an increase rather than a decrease of extracellular adenosine (Latini and Pedata 2001). With the recognition from these papers that ENT proteins are important determinants of nucleoside transport, it is possible that preclinical studies of drugs that are known to be effective blockers of ENT transport function may reveal their therapeutic effectiveness in some neurodegenerative disorders, including ischemic strokes. However, before one can proceed to examining the potential benefits of ENT inhibition in preclinical animal testing, the mechanisms responsible for ENTs regulation, function, pharmacology and therapeutic potentials need further investigation. Here, my goal was to add insight into the regulation ENTs by pharmacologically inhibiting ENTs and CK2, and to determine whether this ENT inhibition results in some neuroprotective mechanisms that limit the subsequent damage to brain tissue during ischemic conditions.

Both ENT1 and ENT2 include consensus sites for PKC, PKA, CK1 and CK2, which suggests that a complex regulation of ENTs by these enzymes likely regulate ENT1/ENT2 in various physiological and pathophysiological conditions (Reyes et al.; Griffiths et al., 1997b). Two splice variants of mouse ENT1 (mENT1) were identified (Kiss et al., 2000; Handa et al., 2001). The Ser-254 site of one variant of ENT1 is one of the accepted protein kinase CK2 phosphorylation consensus sites (Handa et al., 2001). It was found that CK2-regulated phosphorylation of the ENTs mediated their localization on the plasma membrane and/or their trafficking (Stolk et al., 2005). Protein kinase CK2, formerly known as casein kinase II, a cyclic nucleotide-independent serine/threonine protein kinase, is ubiquitously distributed in eukaryotic organisms (Blanquet, 2000; Pinna, 2002; Litchfield, 2003). The other casein kinase, called casein kinase 1 or 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) which could associate to form many distinct

heterotetramers (Blanquet, 2000; Pinna, 2002; Litchfield, 2003). CK2 levels have been observed to be reduced in aged brains and in various neurodegenerative diseases, including Alzheimer's disease and stroke (Blanquet, 2000). CK2 has also been suggested to play a role in neuroprotection during hypoxic insult to the brain (Blanquet, Mariani et al. 2009; Kim, Jung et al. 2009; Lussier, Gu et al. 2014), making it difficult to define and specifically ascribe a role for CK2 in post-ischemic events. It was determined that there is a reduction in CK2 activity 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). Since CK2 is constitutively active and expressed ubiquitously in the brain, it is important to further dissect the role of CK2 in neuroprotection during cerebral ischemic insult. A major challenge in this regard is to identify the important cellular substrates of CK2 that may contribute to the ischemia-induced apoptotic pathways or to neuroprotection. Recently, the AMPAR subunit GluA1 has been shown to be phosphorylated by CK2 (Lussier, Gu et al. 2014) which increases the surface density of AMPARs. Whether this CK2-induced regulation of GluA1 is neuroprotective remains to be determined. From the above discussion on adenosine receptors, AMPARs, ENTs and CK2, it is tempting to speculate that adenosine tone elevation (which could result from ischemic/hypoxic insult or from ENT downregulation and decreased CK2 phosphorylation) and downregulation of A1Rs and AMPARs could be correlated with deficits in synaptic plasticity and increased neuronal death in vulnerable brain areas, such as the hippocampus. The following experimental objectives and methodologies are, therefore, designed to test the general hypothesis that increased adenosine signaling (e.g., in *in vitro* or *in vivo* stroke models) leads to increased downregulation of the neuroprotective, calcium-impermeable AMPAR subunits, which is facilitated by the physical and functional associations of AMPARs with adenosine receptors and ENTs and the activated downstream signaling pathways, including the MAPKs, protein phosphatases, and CK2.

1.17 Rationale, objectives and hypotheses

Rationale:

Ischemic stroke is one of the major causes of death. Although intensive research has been done in the past, little progress has been made in the development of effective clinical agents to prevent subsequent brain damage and death. Extracellular concentration of adenosine increases dramatically during ischemia (Hagberg, Andersson et al. 1987; Phillis, Walter et al. 1987; Dux, Fastbom et al. 1990; Matsumoto, Graf et al. 1992; Latini and Pedata 2001). It is well accepted that A1Rs play an important role in neuroprotection during ischemia (Latini and Pedata 2001; Cunha 2005). In the post-ischemic rat brain, the A1R binding activities decreased (Nagasawa, Araki et al. 1994). Activation of glutamate receptors, including AMPAR, causes neuronal damage after ischemic brain injury (Choi and Rothman 1990; Ying, Weishaupt et al. 1997; Iihara, Joo et al. 2001). Since both levels of adenosine and glutamate increase during ischemic condition, however, the impact of adenosine receptor stimulation on glutamate receptor and its roles in neurotoxicity and neural plasticity require further study.

Extracellular adenosine level is controlled by ENTs. Not only are the adenosine receptors important for adenosine signaling, but adenosine transporters also play a crucial role in this signaling. However, the regulation of ENTs is not well studied in the brain. Therefore, I also aimed to study and explore the regulation of adenosine transporters in normal and ischemic brain by the protein kinase CK2, which has been suggested to target the ENT1 CK2 phosphorylation consensus site at Ser-254 (Kiss, Farah et al. 2000; Handa, Choi et al. 2001). CK2 regulation of ENTs in the brain has never been reported, and my studies will aim to provide a mechanistic link between CK2 activity and function of ENTs, AMPARs, and adenosine receptors in ischemic brain damage.

Elevated extracellular adenosine level was found in aged rats compared to young rats (Sperlagh, Zsilla et al. 1997; Cunha, Almeida et al. 2001; Murillo-Rodriguez, Blanco-Centurion et al. 2004). Cognitive deterioration, memory losses and LTP impairment were also discovered in aged rats (Barnes and McNaughton 1985; Shankar, Teyler et al. 1998; Rex, Kramar et al. 2005; Ritchie, Carriere et al. 2007). Therefore, whether the physical and functional interactions between adenosine receptors and AMPARs become altered

during aging (for example, hyper-adenosine signaling leads to decreased AMPARs in neuronal membranes) remains to be determined. It is also important to determine whether this potential impairment of AMPAR trafficking in aged brains directly leads to changes in synaptic plasticity (for example, impaired plasma membrane targeting of AMPARs leading to decreased surface AMPARs could underlie impaired LTP induction).

Objectives:

1. To determine whether A1R interact with AMPARs to modulate AMPAR trafficking and whether the interaction ultimately leads to A1R-induced persistent synaptic depression (APSD) and stroke damage.
2. To study whether CK2 regulates ENTs and whether this regulation alters the levels or interactions of adenosine receptors and ENTs.
3. To compare the physical and functional interaction between adenosine receptors and AMPARs in young and aged rats.

Hypotheses:

I predict that A1Rs physically and functionally interact with AMPARs to cause A1R-induced persistent synaptic depression (APSD) caused by a prior prolonged stimulation of A1Rs with selective A1R ligands. Previously, our laboratory has reported that A1R-mediated p38 MAPK and JNK activation is important for inhibitory effect of adenosine in rat hippocampus (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). Moreover, activation of p38 MAPK and JNK induced AMPAR endocytosis (Zhu, Qin et al. 2002; Zhu, Pak et al. 2005; Xiong, Kojic et al. 2006). I predict that adenosine receptor activation via A1Rs induces endocytosis of AMPARs through p38 MAPK-JNK pathway. I also predict that A1R-induced endocytosis of AMPARs could cause post-stroke brain damage. Because ENT1 contains CK2 phosphorylation consensus site at Serine-254. I expect CK2 regulates the phosphorylation of ENT1. Elevated extracellular adenosine level has been discovered in aged rats (Rex, Kramar et al. 2005). Having shown recently that prolonged A1R stimulation and focal cortical ischemia results in downregulation of AMPARs in

hippocampus (Chen et al., 2014), I also predict that AMPAR surface expression will be decreased in aged rats compared to young rats. Finally, based on the above discussion and rationale, I predict impairments of LTP will be observed in aged rats due to adenosine receptor-induced endocytosis of AMPARs. These studies, first aimed at elucidating the normal function of adenosine signaling in normoxic environment or in younger animals, will ultimately provide additional insight into the mechanisms of higher adenosinergic signaling in aged brains or in ischemic conditions, which may reveal a new excitotoxic potential of adenosinergic signaling in aging brain or in neurodegenerative diseases, such as stroke. Previous studies described above have assigned a neuroprotective effect of adenosine, acting via A1Rs, in the brain. My results, however, show that adenosine and A1Rs may produce excitotoxic effects when this signaling is significantly prolonged. My studies do not provide definitive proof, but suggests that prolonged adenosine signaling may contribute to the well-known phenomenon of delayed neuronal cell death occurring after ischemic brain damage.

CHAPTER 2

Prolonged adenosine A1 receptor activation in hypoxia and pial vessel disruption focal cortical ischemia facilitates clathrin-mediated AMPA receptor endocytosis and long lasting synaptic inhibition in rat hippocampal CA3-CA1 synapses: differential regulation of GluA2 and GluA1 subunits by p38 MAPK and JNK

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Abstract

Activation of presynaptic adenosine A1 receptors (A1Rs) causes substantial synaptic depression during hypoxia/cerebral ischemia, but postsynaptic actions of A1Rs are less clear. We found that A1Rs and GluA2-containing AMPARs form stable protein complexes from hippocampal brain homogenates and cultured hippocampal neurons from Sprague-Dawley rats. In contrast, adenosine A2A receptors (A2ARs) did not co-precipitate or colocalize with GluA2-containing AMPARs. Prolonged stimulation of A1Rs with the agonist CPA caused *adenosine-induced persistent synaptic depression (APSD)* in hippocampal brain slices, and APSD levels were blunted by inhibiting clathrin-mediated endocytosis of GluA2 with the Tat-GluA2-3Y peptide. Using biotinylation and membrane fractionation assays, prolonged CPA incubation showed significant depletion of GluA2/GluA1 surface expression from hippocampal brain slices and cultured hippocampal neurons. Tat-GluA2-3Y peptide or dynamin inhibitor Dynasore prevented CPA-induced GluA2/GluA1 internalization. Confocal imaging analysis confirmed that functional A1Rs, but not A2ARs, are required for clathrin-mediated endocytosis of AMPARs in hippocampal neurons. Pharmacological inhibitors or shRNA knockdown of p38 MAPK and JNK prevented A1R-mediated internalization of GluA2 but not GluA1 subunits. Tat-GluA2-3Y peptide or A1R antagonist DPCPX also prevented hypoxia-mediated GluA2/GluA1 internalization. Finally, in pial vessel disruption cortical stroke model, a unilateral cortical lesion compared to sham surgery reduced hippocampal GluA2, GluA1, and A1R surface expression, and also caused synaptic depression in hippocampal slices that was consistent with AMPAR downregulation and decreased probability of transmitter release. Together, these results indicate a previously unknown mechanism for A1R-induced persistent synaptic depression involving clathrin-mediated GluA2 and GluA1 internalization that leads to hippocampal neurodegeneration after hypoxia/cerebral ischemia.

2.1 Introduction

Adenosine, a ubiquitous purine nucleoside, plays a putative role as a neuromodulator in both physiological and pathological conditions. Endogenous adenosine is known to be released from neurons and glial cells, and to date, four adenosine receptors have been identified: the A1, A2A, A2B, and A3 receptors (Michaelis, Johe et al. 1988; Dunwiddie and Masino 2001; Fredholm, AP et al. 2001). In cerebral ischemia, adenosine levels rise, rapidly inducing synaptic depression through adenosine A1 receptor (A1R) activation (Fowler 1990; Fowler, Gervitz et al. 2003; Gervitz, Davies et al. 2003), which inhibit presynaptic neurotransmitter release (Lupica, Proctor et al. 1992) by decreasing calcium influx into presynaptic nerve terminals (Dunwiddie and Masino 2001). Adenosine's postsynaptic actions include inhibition of N-methyl D-aspartate receptor (NMDAR)-mediated currents (de Mendonca, Sebastiao et al. 1995), inhibition of adenylate cyclase and stimulation of potassium conductances, all through A1R actions (Siggins and Schubert 1981; Segal 1982; Proctor and Dunwiddie 1983; Haas and Greene 1984). Microscopy evidence shows A1Rs located on somatodendritic hippocampal structures (Ochiishi, Chen et al. 1999), and shown to be highly localized to the active zone and postsynaptic density in hippocampal synapses (Rebola, Pinheiro et al. 2003), suggesting that actions of adenosine are not confined to presynaptic membranes. Some reports also suggest that postsynaptic adenosine receptors (e.g., A2AR and A3R) regulate glutamatergic receptor function and surface distribution (Dias, Ribeiro et al. 2010; Dennis, Jaafari et al. 2011; Dias, Ribeiro et al. 2012).

Alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPARs) are glutamate receptors that form functional tetramers of subunits GluA1-GluA4 (Hollmann and Heinemann 1994; Wenthold, Petralia et al. 1996), and have been implicated in ischemic brain damage which reflects increased expression of GluA2-deficient (Ca^{2+}-permeable) AMPARs on postsynaptic membranes, causing increased permeability to Ca^{2+} (Hollmann, Hartley et al. 1991; Pellegrini-Giampietro, Pulsinelli et al. 1994; Gorter, Petrozzino et al. 1997; Liu, Liao et al. 2006; Liu and Zukin 2007; Kumar and Mayer 2013). Despite this knowledge, it is still unclear whether postsynaptic A1Rs regulate AMPARs in stroke.

During acute administration of the A1R agonist N^6-cyclopentyladenosine (CPA), we observed a profound *adenosine-induced persistent synaptic depression (APSD)* in hippocampal CA3-CA1 synapses and elevation of phosphorylated p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase) in hippocampal membrane fractions (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). Notably, Rap1 and Rap2, GTPases dependent on p38 MAPK and JNK, respectively, mediate NMDAR-dependent AMPAR removal during long-term depression (LTD) (Zhu, Qin et al. 2002; Zhu, Pak et al. 2005). LTD in mouse primary visual cortex was accompanied by activation of p38 MAPK and clathrin-mediated endocytosis of GluA2 AMPARs (Xiong, Kojic et al. 2006).

We therefore propose that A1R-mediated p38 MAPK and JNK activation plays a crucial role in regulating AMPAR trafficking during prolonged hypoxia or an *in vivo* focal cortical small vessel stroke model using type II pial vessel disruption (Wang and Walz 2003; Hua and Walz 2006; Cayabyab, Gowribai et al. 2013). Our results reveal a previously unknown mechanism for APSD involving clathrin-mediated GluA2 internalization via p38 MAPK and JNK signaling observed *after* hypoxic/ischemic insult. Our results also modify the original GluA2 hypothesis of excitotoxicity (Pellegrini-Giampietro, Gorter et al. 1997) in that selective activation of A1Rs can mediate GluA2-containing AMPAR internalization in vulnerable regions, including the hippocampus, representing an important mechanism of ischemic damage with therapeutic potential.

2.2 Materials and Methods

2.2.1 Hippocampal slice preparation and treatments

Hippocampal slices from male Sprague-Dawley rats (P21-28 days) were anaesthetized with halothane and rapidly decapitated according to protocols approved by the University Committee of Animal Care and Supply at the University of Saskatchewan. The brains were extracted and immediately placed into ice-cold oxygenated dissection medium. Hippocampal slices (400μm thick) were cut using a vibrating tissue slicer (VT1200S, Leica, Nussloch, Germany) and maintained for 60-90 minutes in artificial

cerebrospinal fluid (ACSF) before performing electrophysiological recordings or biochemical analysis. Recipes for ACSF and dissection solutions and details of recording conditions were described previously (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). All experiments were conducted at room temperature.

2.2.2 Pial Vessel Disruption (PVD) as a model of small-vessel stroke

Class II size pial vessel disruption (PVD) has been shown to induce a focal cortical lesion that, within 3 weeks of surgery lesion, leads to lacunar infarction-like fluid-filled cyst that does not extend to the corpus callosum. This fluid-filled cavity is tightly surrounded by a barrier consisting of processes from reactive astrocytes – the hallmark of lacunar infarctions (Hua et al., 2006, 2008). The genesis of such a lacuna (cavitation) has been studied in more detail previously. It has been found that treatment with minocycline or the specific matrix metalloproteinase inhibitor batimastat, an experimental anti-cancer drug, prevents cavitation and leads to a lesion filled with reactive astrocytes and no barrier (Cayabyab et al., 2013). The procedure is described in detail in previous studies (for further details, see: (Wang and Walz 2003; Hua and Walz 2006; Hua and Walz 2006; Cayabyab, Gowribai et al. 2013)). Briefly, Sprague-Dawley rats under 2% isoflurane anesthesia and buprenorphine treatment for pain management received a craniotomy with 5-mm-diameter on the right and rostral side of the bregma adjacent to the coronal and sagittal sutures. After opening of the dura the class II pial vessels were disrupted with fine-tipped forceps. The piece of bone was placed back and the wound was closed with a clip. Sham animals received the same treatment with dura removal but no vessel disruption. This procedure including the recovery period of the animal was approved under permit 20020024 by the Animal Research Ethics Board of the University of Saskatchewan.

To investigate the impact of a remote focal ischemic injury on hippocampal signaling we used this modified pial vessel disruption model. The modification consists of the disruption of the class II medium vessels only and not the class I large vessels. We used this *in vivo* animal stroke model because it has distinct advantages over other models.

For example, this PVD model is a small-vessel stroke model that produces permanent damage to class II size vessels (i.e., a non-reperfusion model), and the cortical lesion volumes can be reliably reproduced and have similarities to a lacunar infarction (Wang and Walz 2003; Hua and Walz 2006). In contrast, most focal or global stroke models involve transient occlusion of large vessels, such as the middle cerebral arteries or carotid arteries (Pellegrini-Giampietro, Zukin et al. 1992; Gorter, Petrozzino et al. 1997; McBean and Kelly 1998; Prosser-Loose, Verge et al. 2010; Tu, Xu et al. 2010) and the cerebral ischemic damage often encompasses large volumes of brain regions. Our PVD model results in an approximately 1mm^3 cortical lesion volume and is a more subtle small vessel injury in the cerebral cortex (Wang and Walz 2003). Previous cortical devascularization studies have shown that focal cortical ischemia affects the hippocampus by altering hippocampal synaptic transmission (Ramos, Rubio et al. 2004) and increasing expression of both c-fos (Herrera and Robertson 1990) and nerve growth factor (Figueiredo, Pluss et al. 1995), and adenosine has been implicated in the increased expression of both of these regulatory factors in other brain regions (Rudolphi 1995; Svenningsson, Fourreau et al. 1999). Since stroke in humans (Laghi Pasini, Guideri et al. 2000) and transient middle cerebral artery occlusion in large vessel animal stroke models (Matsumoto, Graf et al. 1992) have been associated with transient surges in global brain adenosine levels, we hypothesized that brain adenosine elevation occurring in our non-reperfusion PVD stroke model could affect AMPAR trafficking. Interestingly, two days post PVD we found MMP-2 elevation on both the ipsilateral and contralateral side of the PVD cortical lesion (Cayabyab, Gowribai et al. 2013). Therefore, in the current study we performed morphological, biochemical and electrophysiological analyses of hippocampal tissue taken from both ipsilateral and contralateral sides of the lesion in sham- and PVD-treated animals. We analyzed the effects of PVD on neurodegeneration using Fluoro-Jade B staining and confocal imaging, on the changes in adenosine tone using fEPSP recordings, and on alterations in the surface levels of both GluA2 and GluA1 AMPARs and adenosine A1 and A2A receptors using biotinylation and western blot analyses as described below. Subsequent results are consistent with PVD inducing elevation of adenosine tone, downregulation of AMPARs and A1Rs, upregulation of A2ARs, and increased Fluoro-Jade B staining in hippocampus.

2.2.3 Biochemical studies

For biotinylation experiments, hippocampal slices or 7-day cultured hippocampal neurons were incubated with 1.2μM tetrodotoxin (TTX, Thermo-Fisher Scientific Inc., Rockford, IL) to prevent glutamate release induced by treatments with the A1R antagonist DPCPX, which could confound the direct effects of CPA on GluA2 and GluA1 AMPAR internalization. In addition to TTX, bicuculline (50μM, Thermo-Fisher), strychnine (1μM, Thermo-Fisher) and D-APV (50μM, Thermo-Fisher) were applied for 20-30 min, to block $GABA_A$ currents, glycine receptors, and N-methyl-D-aspartate (NMDA) receptors, respectively. After CPA treatments (500nM, 45min), slices or neurons were cooled to 4°C (20-30min) and washed with ice-cold ACSF before biotinylation. Hippocampal brain slices or neurons were incubated with 1mg/ml NHS-SS-Biotin (Pierce, Thermo-Fisher Scientific Inc., Rockford, IL) at 4°C for 45 min. Quenching with glycine buffer containing 192 mM glycine, 25mM Tris, pH 8.3 stopped the reaction. Slices were then transferred into lysis buffer containing protease inhibitors and 1% NP-40 detergent. Lysis buffer contained 50mM Tris (pH8.0), 150mM NaCl, 1mM EDTA (Ethylenediaminetetraacetic acid), 1mM NaF; and the following protease inhibitors: 1mM PMSF (phenylmethylsulfonyl fluoride), 10μg/μL aprotinin, 10μg/mL pepstatin A, 10μg/mL leupeptin, 2mM Na_3VO_4, 20mM sodium pyrophosphate, 3mM benzamidine hydrochloride, and 4mM glycerol 2-phosphate. After determining the protein concentrations using the Bradford Assay with the DC Protein assay dye (Bio-Rad, Mississauga, ON, Canada), equal amounts of protein lysates (200-500μg) were diluted in lysis buffer and biotinylated proteins were incubated overnight with the streptavidin beads (Thermo-Scientific). The beads were then washed 4-6 times the next day with lysis buffer containing 0.1% NP-40. The proteins were eluted by adding 50μl of 2X Laemmli sample buffer (Bio-Rad) and boiling the samples at 95°C for 5min. The proteins were the separated by running the samples through 10% polyacrylamide gels and the resulting blots were probed with the appropriate primary and secondary antibodies. Enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology Inc., Dallas, TX) was used to visualize the labeled proteins.

Coimmunoprecipitation was performed to examine interactions between adenosine receptors and AMPARs by incubating 500μg of extract from hippocampal homogenates with mouse, goat or rabbit IgG (1h, 4°C). Then mouse, goat or rabbit IgG agarose beads (Sigma) were added to the homogenates for an additional 1h or overnight. After this pre-clearing stage, the agarose beads were removed by pulse spinning at 6000rpm for 5s, and the supernatant was subsequently reacted with the appropriate immunoprecipitating antibody overnight at 4°C. The A1 receptor and other proteins were immunoprecipitated with a polyclonal goat anti-A1 receptor (5μg, Santa Cruz Biotechnology), a polyclonal rabbit anti-A1 receptor (5μg, Sigma), a mouse monoclonal GluA2 antibody (2μg, EMD Millipore, Billerica, MA), a rabbit anti-A2A receptor antibody (5μg, Sigma), or a rabbit anti-GluA1 antibody (5μg, Millipore). After overnight incubation of lysates with a polyclonal rabbit or monoclonal mouse antibody for the target listed above, the antigen was captured by incubation of immune complexes for 4h at 4°C with agarose beads conjugated to secondary antibody (rabbit, mouse or goat anti-IgG). Agarose beads were then collected by pulse spins, and washed four times with wash buffer (lysis buffer listed above containing 0.1% NP-40). Proteins from the agarose beads were detected by western blotting. The antibody dilutions were as follows: polyclonal rabbit anti-A1 receptor or anti-A2A receptor (1:1000, Sigma), rabbit anti-GluA1 (1:1000, Millipore), rabbit anti-GluA1 (pSer831) (1:1000, Millipore), rabbit anti-GluA1 (pSer845) (1:1000, Millipore), and mouse anti-GluA2 (1:1000, Millipore). To normalize the protein bands from the membrane fractions, we used a monoclonal mouse anti-GAPDH (1:2000, Millipore) to quantify signals of GluA2 and GluA1 bands. Whole hippocampal lysate blots or blots containing biotinylated proteins were re-probed with anti-β actin antibody (1:1000, Sigma). Labeled protein bands were visualized using ECL reagent (Santa Cruz).

In some experiments, the membrane fractions from hippocampal slices were separated by centrifugation at 13,000g for 1h at 4°C by omitting the detergent (NP-40) from the solubilization buffer. The proteins from the particulate (membrane) fraction were resolved in normal solubilization buffer after removal of the cytosolic fraction. Hippocampal homogenates were diluted with Laemmli sample buffer, boiled for 5min and resolved in 10% polyacrylamide gel; then they were electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The amount of protein loaded into the gels was

consistent across all experiments where 50μg were loaded for total lysates and 300-500μg of total lysates were used for immunoprecipitation. After blocking with 5% nonfat milk in TBST for 1h or overnight, the membranes were incubated with primary antibody in 5% nonfat milk in TBST containing 0.025% sodium azide overnight at 4°C. The PVDF membranes were washed four times with TBST for 15min and then incubated with a mouse, goat, or rabbit horseradish peroxidase-conjugated secondary antibody against IgG (1:1000; Santa Cruz) in 5% nonfat milk. After three to four 15min washes with TBST, proteins were visualized using ECL (Santa Cruz).

2.2.4 Hippocampal neuron culture, immunocytochemistry, and confocal imaging

Rat hippocampal neurons were cultured as described by others (Kaech and Banker, 2006) and used for immunocytochemistry 12-15 days after plating. In brief, low-density hippocampal neurons ($5x10^4$ cells/35mm culture dishes) from 17-18 day old embryonic rat brains were grown on polylysine-coated coverslips, which were suspended above a one-week-old astrocyte feeder layer. For immunocytochemistry, the hippocampal neurons were treated with pharmacologic agents TTX (1.2μM), bicuculline (50μM), strychnine (1μM) and D-APV (50μM) for 20-30 min, to block neural activity, $GABA_A$ currents, glycine receptors, and N-methyl-D-aspartate (NMDA) receptors, respectively, and then incubated for 1h with the A1R antagonist DPCPX (500nM, Sigma), the A2A receptor antagonist SCH 58261 (30nM), the inhibitor of GluA2 endocytosis Tat-GluA2-3Y peptide (2μM) or the scrambled Tat-GluA2-3Y peptide (2μM) before a final 45 min incubation with the A1R agonist CPA (500nM). The active Tat-GluA2-3Y peptide consists of the following amino acid sequence: YGRKKRRQRRR-^{869}YKEGYNVYG^{877}, where Tat is YGRKKRRQRRR (the cell penetrating amino acid peptide sequence contained within the protein transduction domain of HIV gene called Tat), and ^{869}YKEGYNVYG^{877} represents a GluA2 C-terminal amino acid sequence that interacts with the endocytic protein AP2 thus preventing GluA2 internalization (Ahmadian et al., 2004). The Tat-GluA2-3Y peptide and its scrambled version (scrambled Tat-GluA2-3Y: YGRKKRRQRRR-VYKYGGYNE) were purchased from GL Biochem Ltd. (China). The

A2A receptor agonist CGS 21680 (10nM) was applied to investigate the relationship between A2A receptor and possible internalization of AMPARs. After fixation, neurons were blocked for 1h at RT with PBS containing 5% bovine serum albumin (BSA, Sigma).

To assess the effects of A1R or A2AR stimulation on AMPAR surface expression, hippocampal neurons were washed three times with ice-cold phosphate buffered saline (PBS), fixed with 4% paraformaldehyde, and then blocked for 1h at RT with PBS containing 5% BSA. Surface proteins of neurons were labeled by overnight incubation (at 4°C) with rabbit anti-GluA1 (extracellular epitope, Alomone Labs, Jerusalem, Israel) or mouse anti-GluA2 (extracellular epitope, Millipore) antibody diluted at 1:250 in blocking buffer followed by three brief washes (10min each) then incubated with Alexa Fluor 555-conjugated goat anti-rabbit or Alexa Fluor 488-conjugated donkey anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA) at 1:1000 for 1 h at RT. All the neurons were labeled with chicken anti-rat MAP2 antibody (1:2000, Abcam, Cambridge, UK) and Alexa Fluor 633-conjugated anti-chicken secondary antibody at 1:1000 and Hoechst (Sigma) after permeabilization with 0.25% Triton X-100 and blocking with 5% BSA. Lastly, the coverslips were mounted on newly cleaned slides using Prolong Gold Antifade Reagent (Invitrogen) and observed with a LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). To demonstrate possible colocalization between GluA2 and GluA1 AMPARs and either A1Rs or A2ARs, hippocampal neurons were permeabilized with 0.25% Triton-X100 before subsequent incubation with primary and secondary antibodies (see Figure 2.3.2 legends for further details of primary and secondary antibody combinations used). Rabbit polyclonal A1R or A2AR antibodies (1:100, Sigma), goat anti-A1R (1:100, Santa Cruz), and mouse anti-A2AR (1:100, Santa Cruz) were used. All neurons were also co-labeled with chicken anti-MAP2 (1:2000).

The images were acquired using a Zeiss Plan-Apochromat 63X/1.4 oil objective lens and analyzed with the Zeiss Zen 2009 software (version 5.5 SPI). The 10μm dendritic lengths located 5μm away from the cell soma were included in the analysis of GluA2 and GluA1 surface expression, and identical acquisition parameters were used for a given set of labeled neurons without over- or under-saturation of the acquired signals. For analyses of AMPAR and adenosine receptor colocalization in dendrites, the image window of

~10μm dendritic length by ~2μm dendritic width at 0.06μm pixel resolution was used for the regions of interest comparisons for the A1R/GluA2, A1R/GluA1, A2AR/GluA2, and A2AR/GluA1 groups. Images were exported as 8 bit TIFF files, and the degree of colocalization between fluorescent probes was quantified by using the Intensity Correlation Analysis plug-in of ImageJ software (downloaded from NIH, version 1.44f), which reported the Pearson's correlation coefficients. Negative Pearson's correlation coefficients indicate the two signals do not colocalize, while values closer to 1 indicate strong colocalization between the two particles. The average signal intensities or Pearson's correlation coefficients from two to four dendritic processes from a given neuron were determined, and the n-values reported in the summary bar charts refer to the number of cells analyzed from at least three different experiments. Different lab personnel were involved in preparing the immunocytochemical slides and performing confocal analyses to reduce bias. Data are presented as mean±SEM. Group results were analyzed by one-way analysis of variance (ANOVA) with Student-Neuman-Keuls post hoc test when comparing more than two treatment groups. $P>0.05$ was considered not significant (NS). For transfection experiments, hippocampal neurons were transfected with 1μg of p38α MAPK shRNA, JNK1 shRNA or control plasmid A (Santa Cruz) and 2μL Lipofectamine 2000 (Invitrogen). Two days post-transfection, hippocampal neurons were treated with 500nM CPA for 45min followed by immunocytochemistry as described above.

2.2.5 Fluoro-Jade B staining

Sprague-Dawley rat brains were prepared and sectioned as described previously (Cayabyab et al., 2013). In brief, anesthetized rats were intracardially perfused with 4% paraformaldehyde in PBS for 30 min. Following perfusion, brains were removed and post-fixed in 4% paraformaldehyde in PBS overnight. Brains were then stored in 30% sucrose (w/v) in 0.1M PBS for additional three days. The brains were then frozen in liquid nitrogen in Tissue-Tek OCT mounting medium, and 30 μm coronal sections of hippocampus were cut with cryostat. Sections were subsequently collected on paraffin coated slides and allowed to air dry. Sections were immersed in 70% ethanol, washed three times with

ultrapure water (1 min each), and then soaked in 0.06% KMNO4 (15 min). After three 1min- washes with ultrapure water, sections were subsequently stained with 0.001% Fluoro-Jade B (Chemicon International, Temecula, CA) for 20 min. Slides were subsequently washed three times in ultrapure water (1 min each) and allowed to dry overnight. Slides were then rinsed in Xylene and coverslips were mounted using Prolong Gold antifade reagen (Invitrogen). Digital images were obtained with Zeiss LSM 700 (Carl Zeiss) using a 20X objective for the hippocampal montages and 63 Oil immersion lens for the magnified regions of the hippocampal pyramidal body layers. Three sham and three PVD rat brains were used for Fluoro-Jade B staining.

2.2.6 Electrophysiological studies

Field excitatory postsynaptic potentials (fEPSPs) were evoked by orthodromic stimulation of the Schaffer collateral pathway using a bipolar tungsten-stimulating electrode. Glass micropipettes filled with ACSF (resistance 1-3MΩ) were used to measure CA1 fEPSPs in *stratum radiatum*. The fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1-100Hz, digitized at 10kHz using a Digidata 1320A interface board (Axon Instruments, Foster City, CA), and transferred to a computer for analysis. Data were analyzed using Clampfit 9.0 (Axon Instruments). Baseline synaptic responses were established by evoking fEPSPs every 30s (0.03 Hz) for at least 20min. Paired pulses separated by 50ms were also evoked every 30s to assess changes in presynaptic function in control, CPA-treated, sham or PVD hippocampal slices. The fEPSP slope was normalized to the mean of the 20 sweeps (10min) immediately preceding drug perfusion. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the standard error of the mean (SEM). Sample traces are the average of 5 sweeps from a recording that was included in the plot of the mean normalized fEPSP slope. All bar graphs show the mean normalized percent inhibition from baseline ± SEM. Statistical significance was assessed using one-way analysis of variance with Student-Neuman-Keuls post hoc test.

2.2.7 Drug inhibitors

N^6-cyclopentyladenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Sigma-Aldrich. Baclofen, 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS 21680), okadaic acid, fostriecin, Dynasore, and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH 58261) were obtained from Tocris Biosciences. SB203580, SB202474, JNK II inhibitor (also called SP600125), and JNK inhibitor II negative control (N^1-methyl-1,9 pyrazoloanthrone) were obtained from Calbiochem (San Diego, CA). All drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma) before being added to ACSF. The final concentration of DMSO was always < 0.1%.

2.2.8 Statistical analysis

Densitometry was performed using Quantity 1 (Bio-Rad) and ImageJ (public domain). A single analysis of variance was performed to obtain the overall significance of the treatments followed by a post-hoc Student-Newman-Keuls. Significances for the multiple comparisons tests are indicated in the summary bar charts as follows: *P<0.05, **P<0.01, and ***P<0.001. Student's paired *t*-test was also used when comparing two treatment groups. All statistical tests were performed with GraphPad Instat3 version 3.00 for Windows 97 (GraphPad Software, San Diego, CA).

2.3 Results

2.3.1 GluA2 and GluA1 AMPARs physically interact with A1Rs, but not with A2A receptors

Physical interactions between different transmembrane G-protein-coupled receptors (GPCRs) and ionotropic glutamate receptors are known to exist (Salter 2003; Lee and Liu 2004). For example, D2 dopamine receptors exhibit an indirect biochemical interaction with GluA2-containing AMPARs, which causes downregulation of AMPAR surface expression (Zou, Li et al. 2005). Direct interactions between two or more GPCRs are also possible (Angers, Salahpour et al. 2002). For example, it has been shown that A1Rs may form heterodimers with A2ARs (Ciruela, Ferre et al. 2006). Thus, it is reasonable to propose a possible association of AMPARs with the GPCR A1Rs and A2ARs, which are the most abundant of the four known adenosine receptors in the brain (Dunwiddie and Masino 2001). We initially sought to characterize this interaction in hippocampal slices and cultured hippocampal neurons.

We performed coimmunoprecipitation experiments to determine whether the adenosine A1R exists in the same signaling protein complex as GluA2 and GluA1 in the rat hippocampus. We found that both GluA2 and GluA1 formed stable complexes with A1Rs (Figure 2.3.1A-B, left panels), and the reverse immunoprecipitation confirmed the interaction of GluA2 and GluA1 in the A1R immunoprecipitates (Figure 2.3.1A-B, right panels). However, our coimmunoprecipitation studies did not reveal an association between adenosine A2A receptors with either GluA2 or GluA1 AMPARs (Figure 2.3.1C-D). These results indicated that the inhibitory A1Rs, but not the excitatory A2A receptors, are specifically localized in the same protein complex as GluA2-containing AMPARs. These interactions could certainly contribute to the modulation of the AMPAR function and subcellular distribution.

Figure 2.3.1

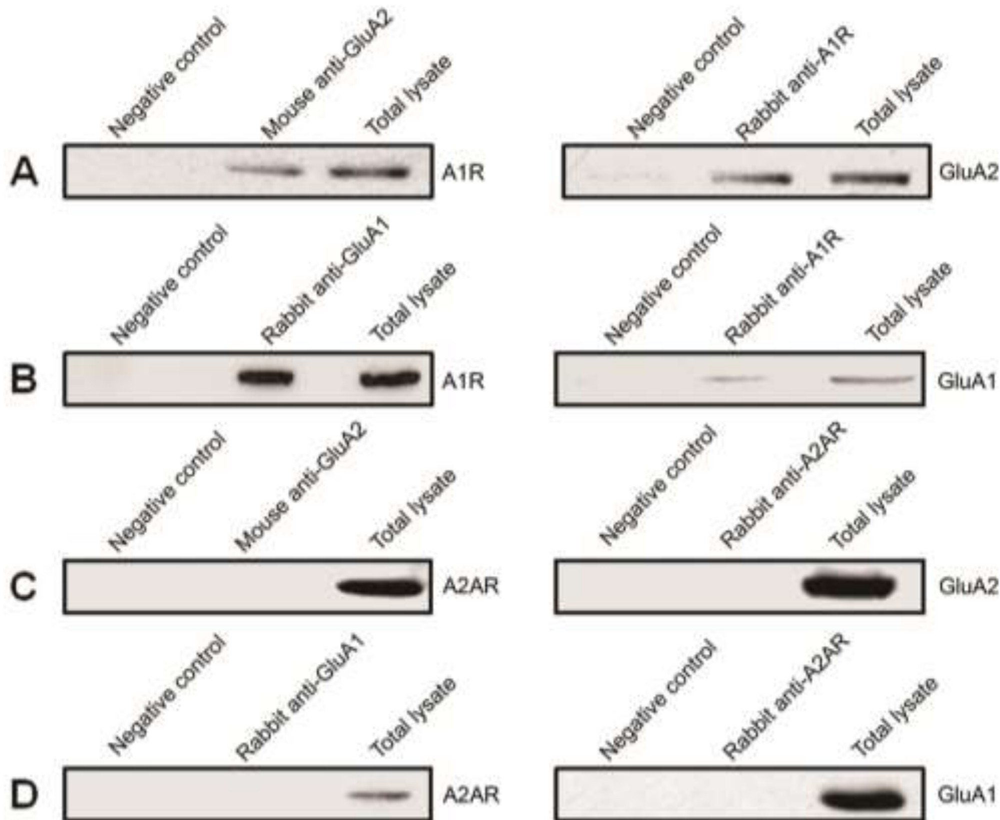


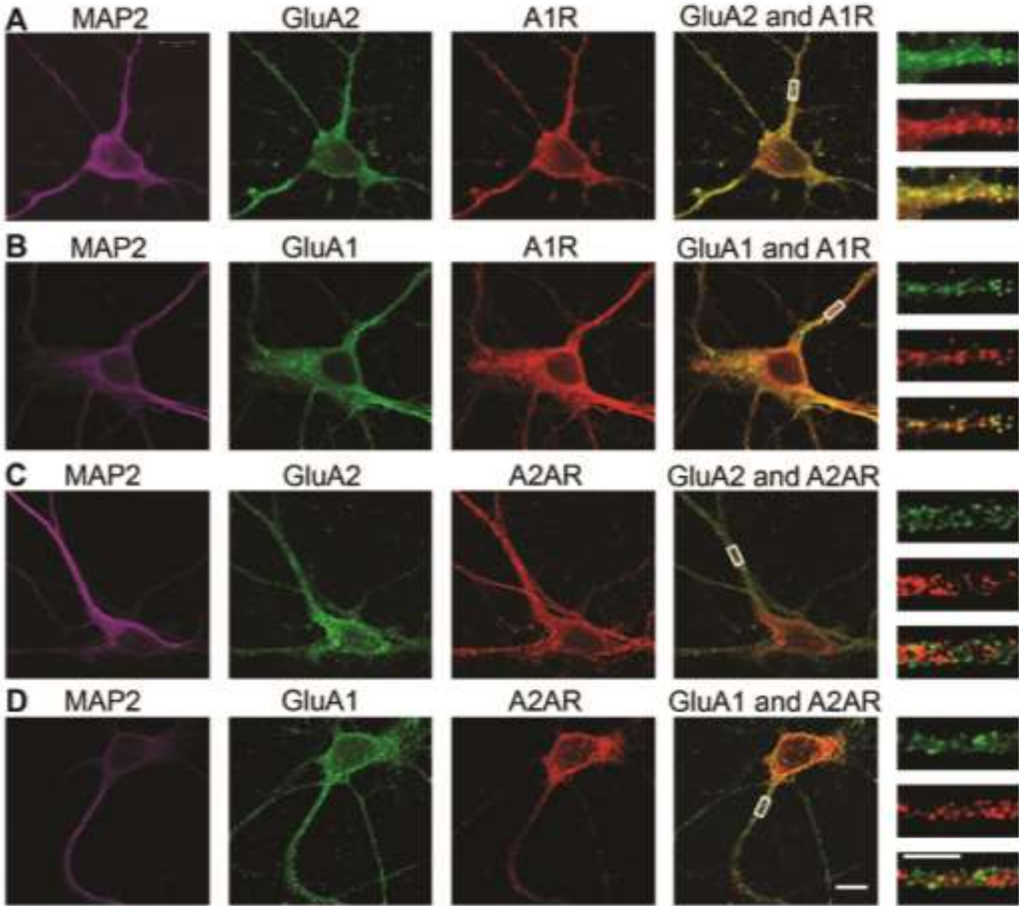
Figure 2.3.1 AMPARs physically interact specifically with A1Rs (A1Rs). (A) GluA2-containing AMPAR immunoprecipitated complexes from rat hippocampus contained A1Rs, and A1R immunoprecipitated complex contained GluA2 subunits. (B) GluA1 immunoprecipitate from rat hippocampal tissue also contained A1Rs, and the A1R immunoprecipitate likewise contained GluA1 subunits. (C) Coimmunoprecipitation of GluA2 did not include adenosine A2A receptors (A2ARs), and A2AR antibody did not immunopreciptitate GluA2-containing AMPARs. (D) GluA1 immunoprecipitate did not contain A2ARs, and A2AR immunoprecipitate did not contain GluA1 subunits. These forward and reverse coimmunoprecipitation studies are from at least three independent experiments, using hippocampal brain lysates from P18-28 day rats. The molecular weights of the specific bands on the blots were estimated from pre-stained protein standards, and are as follows: A1R (37 kDa), A2AR (45 kDa), GluR1 (106kDa), and GluR2 (102 kDa). 50 µg of protein was loaded into gels as positive control for western

blot and 500 μg of protein was as intial proteins for IP. Antibody sources and dilutions are indicated in the Materials and Methods.

2.3.2 GluA2- and GluA1-containing AMPARs colocalize with A1Rs, but not with A2A receptors, in cultured hippocampal neurons

To further confirm the potential interaction of A1Rs with GluA2 and GluA1 AMPARs, we used immunocytochemistry and confocal imaging to test whether A1Rs and GluA2-containing AMPARs colocalized in cultured hippocampal neurons. Hippocampal neurons were cultured for 12 days, then fixed with 4% paraformaldehyde and permeabilized with detergent to label surface and intracellular localizations of proteins. Mouse anti-GluA2 or mouse anti-GluA1 was used with rabbit anti-A1R antibodies, or mouse anti-GluA1 and rabbit anti-A2AR antibodies, followed by incubation with appropriate fluorescent secondary antibodies for double staining of cultured neurons. Visualization of neuronal morphology was facilitated by subsequent immunolabeling with chicken anti-MAP2 antibody and secondary antibody. Immunocytochemical identification of GluA2/GluA1 is shown in green, and A1R/A2AR labeling is in red. Merging the GluA2 or GluA1 AMPAR with the A1R images revealed overlapping regions of colocalization, which is shown as yellow pixels (Figure 2.3.2A-B). In contrast, merging GluA2 and GluA1 with A2ARs produced very few yellow pixels were visible, suggesting little colocalization of A2ARs with GluA2 or GluA1 AMPARs in hippocampus (Figure 2.3.2C-D). Quantification of overlapping A1Rs and GluA2 or A1Rs and GluA1 AMPARs revealed a significant colocalization of A1Rs with GluA2 and GluA1 AMPARs (Figure 2.3.2E). For example, the Pearson correlation coefficients for A1R/GluA2 colocalization were 0.81±0.02 (arb. units) (n=14) compared with 0.08±0.01 for A2AR/GluA2 colocalization (n=14, $P<0.001$). Together, the above biochemical and confocal imaging results indicated that A1Rs, but not A2ARs, specifically formed a physical complex with GluA2 and GluA1 AMPARs and are localized in similar dendritic and somatic compartments of hippocampal neurons.

Figure 2.3.2



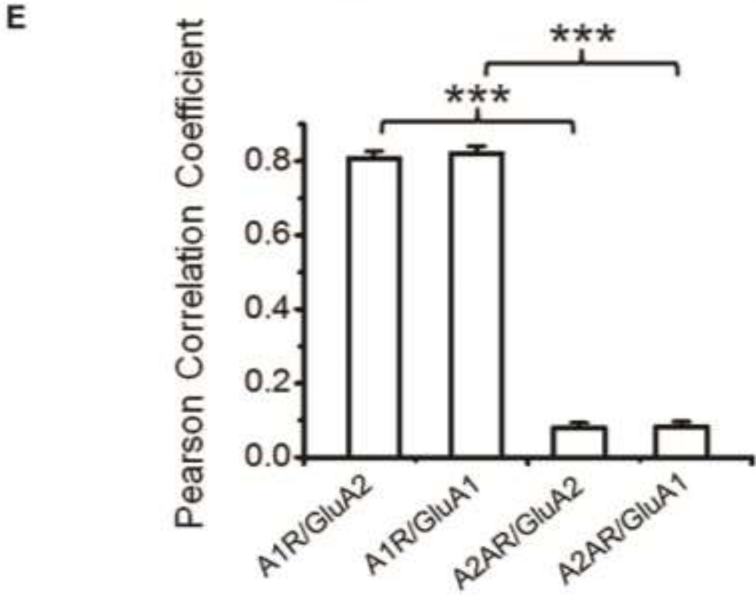


Figure 2.3.2 Co-localization of GluA2 and GluA1 with A1R, but not with A2AR, in rat hippocampal neurons. After membrane permeabilization, hippocampal neurons were triple co-labeled with the following primary antibodies: 1) chicken anti-MAP2 (Abcam); 2) mouse anti-GluA2 or rabbit anti-GluA1 (both from Millipore); and 3) goat anti-A1R (Santa Cruz), mouse anti-A2AR (Santa Cruz), rabbit anti-A1R (Sigma), or rabbit anti-A2AR (Sigma). Secondary antibodies used were conjugated to Alexa 633 (for MAP2, magenta panels), Alexa 488 (for GluA2 or GluA1, green panels), and Alexa 555 (for A1R or A2AR, red panels). (A-B) Shows the immuno-labeling of MAP2 (magenta panels), GluA2 (Millipore) (A) or GluA1 (Millipore) (B) (green panels), A1R (Sigma) (A) or A1R (Santa Cruz) (B) (red panels), GluA2 or GluA1 merged with A1R (fourth panels from left), and magnified views of the dendrites indicated by the respective rectangular regions (last panels). A1Rs colocalized with GluA2 (A) or GluA1 (B) around the somas and dendrites, as shown by the highly intense yellow pixels in the merged fourth and fifth panels. (C-D) Shows the immuno-labeling of MAP2 (magenta panels), GluA2 (Millipore) (C) or GluA1 (Millipore) (D) (green panels), A2AR (Sigma) (C) or A2AR (Santa Cruz) (D) (red panels), GluA2 or GluA1 merged with A2AR (fourth panels from left), and magnified views of the dendrites indicated by the respective rectangular regions (last panels). A2AR did not colocalize with either GluA2 (C) or GluA1 (D) around the somas and dendrites, as shown by the absence of yellow pixels in the merged fourth and fifth panels. Large and small scale bars are 10μm and 5μm, respectively. (E) Colocalization of adenosine receptors with AMPARs was quantified by determining the Pearson correlation coefficients. Dendritic lengths (10μm) taken 5μm away from somas from different staining experiments were used for co-localization analysis. The values in bars represent the mean ± SEM, N=14 neurons each column (from 4 independent hippocampal neuronal cultures, 3-4 representative neurons included per culture), ***$p<0.001$.

2.3.3 Stimulation of adenosine A1 receptor triggers GluA2 and GluA1 AMPAR internalization via clathrin-mediated and dynamin-dependent endocytosis

Next, we tested whether this specific physical association of A1Rs with GluA2 and GluA1 AMPARs can functionally modify AMPAR trafficking, which is important for

excitation of neurons (Bredt and Nicoll 2003; Malinow 2003). To determine whether selective activation of A1Rs could alter the trafficking of GluA2 and GluA1 AMPARs, we performed surface biotinylation of primary cultures of hippocampal neurons followed by western blotting to track changes in GluA2 and GluA1 surface levels. The results showed that stimulation of A1Rs with the A1R-selective agonist CPA (500nM, 45min) caused a significant decrease in GluA2 and GluA1 surface levels (Figure 2.3.3A-B).

To determine whether this inhibitory effect requires the clathrin-mediated endocytosis pathway, we pre-incubated the neurons with Tat-GluA2-3Y peptide, which has been used by other labs to block the clathrin-mediated endocytosis of GluA2 (Ahmadian, Ju et al. 2004; Brebner, Wong et al. 2005; Xiong, Kojic et al. 2006). Pre-incubation with Tat-GluA2-3Y peptide prevented the A1R-induced decrease in GluA2 and GluA1 surface expression in cultured hippocampal neurons (Figure 2.3.3A-B, third column). The scrambled Tat-GluA2-3Y peptide did not alter CPA-induced GluA2 and GluA1 surface levels. Similar results were obtained using membrane fractionation of hippocampal brain slices. That is, GluA2 levels in membrane fractions were as follows: Control (DMSO) 100%, CPA alone 78.6±2.7% ($P<0.01$ compared to control), CPA + Tat-GluA2-3Y 93.5±3.1% ($P>0.05$), and CPA + Scrambled Tat-peptide 72.4±4.9% ($P<0.01$ compared to control). All signals were normalized to GAPDH, with N=5 independent experiments ($P<0.0001$, one-way ANOVA). Not surprisingly, we observed a similar pattern of changes in the GluA2 and GluA1 surface distribution in hippocampal neuronal cultures after A1R stimulation (Figure 2.3.3A-B), since both GluA2 and GluA1 are known to form heteromers, and GluA2 and GluA1 heteromeric AMPARs are the most widely expressed subunits in the hippocampus (Wenthold, Petralia et al. 1996; Sans, Vissel et al. 2003; Cull-Candy, Kelly et al. 2006). However, upon closer inspection, it is apparent that the levels of CPA-induced GluA1 internalization were higher (~50%, Figure 2.3.3B bottom) compared to those for GluA2 internalization (~30%, Figure 2.3.3A bottom). When PVDF membranes of biotinylated proteins and total lysates were re-probed with β-actin antibody, the β-actin was only found in total lysates and that cytosolic proteins were undetectable in blots containing biotinylated surface proteins. Thus, these results suggest that stimulation of A1R induces clathrin-mediated endocytosis of GluA2-containing AMPARs in hippocampal neuronal cultures.

Previous reports also suggested that AMPAR internalization could be mediated by dynamin-dependent endocytosis (Carroll, Beattie et al. 1999; Luscher, Xia et al. 1999; Man, Lin et al. 2000; Xiong, Kojic et al. 2006). Dynamin is critical for the vesicle formation during clathrin-mediated endocytosis (Henley, Cao et al. 1999). Since the use of the Tat-GluA2-3Y peptide implicated the clathrin-mediated pathway for A1R-mediated AMPAR endocytosis, we then determined whether inhibition of dynamin function with Dynasore, a dynamin antagonist (Macia, Ehrlich et al. 2006; Newton, Kirchhausen et al. 2006), would also blunt the level of CPA-induced AMPAR endocytosis. Pre-incubation of hippocampal slices with Dynasore (100μM) for 1h prior to CPA stimulation and subsequent biotinylation of surface-expressed proteins revealed that Dynasore did indeed prevent A1R-induced internalization of both GluA2 and GluA1, as summarized in bar charts in Figure 2.3.3C-D. Together, the results above demonstrate that prolonged A1R stimulation led to a clathrin-mediated and dynamin-dependent internalization of GluA2 and GluA1 AMPARs in both hippocampal neurons and hippocampal brain slices.

Figure 2.3.3

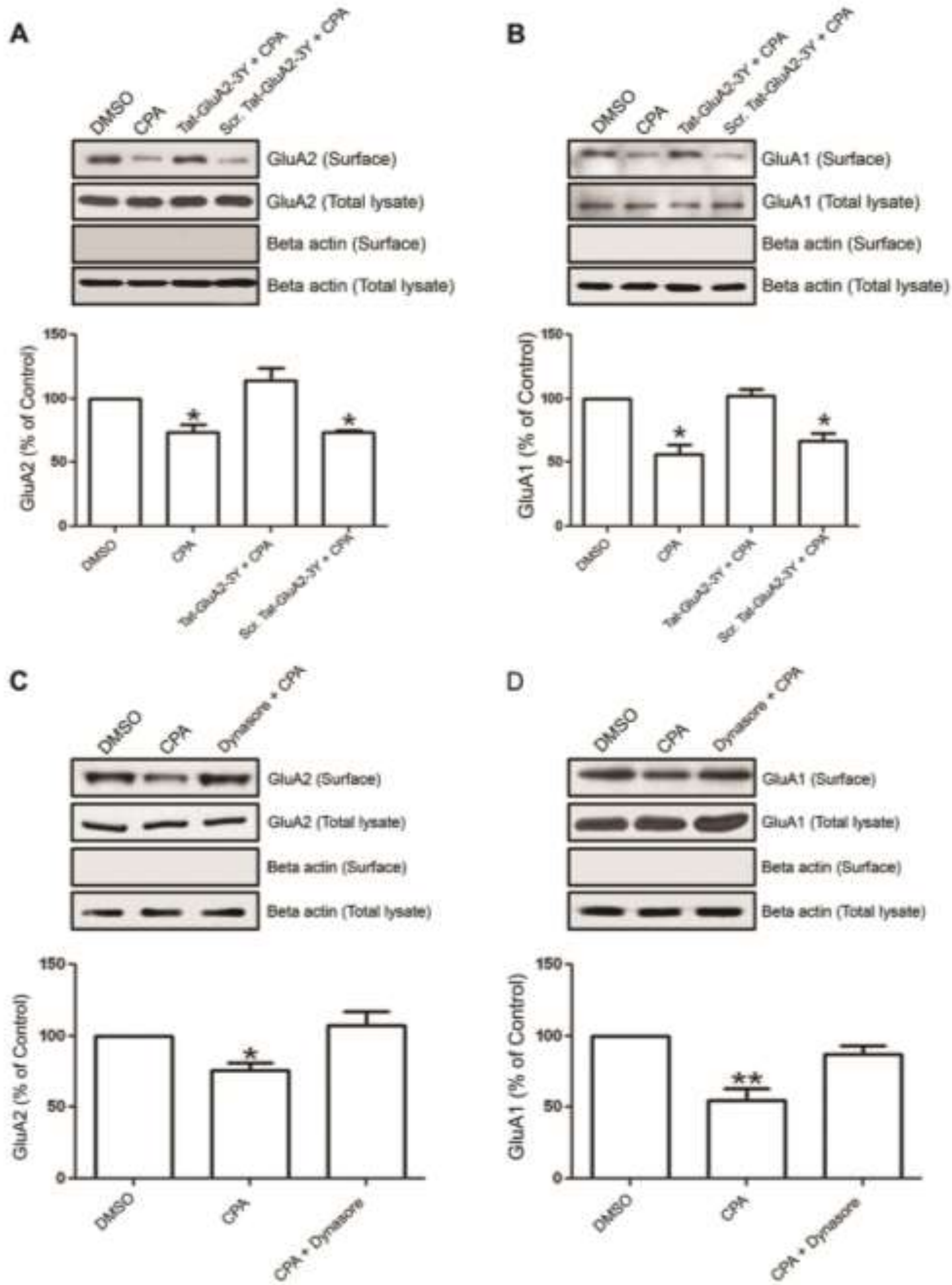


Figure 2.3.3 Prolonged A1R stimulation caused clathrin-mediated AMPAR endocytosis. Surface expression of GluA2 decreased by A1R stimulation in cultured hippocampal neurons. (A) Cell-surface biotinylation of hippocampal neurons showed a significant decrease in GluA2 surface expression after CPA treatment (500nM, 45min) compared with DMSO (Control). This effect was abolished with Tat-GluA2-3Y (2μM) peptide, which specifically blocks clathrin-mediated GluA2 endocytosis, but the scrambled Tat-GluA2-3Y peptide did not prevent the inhibitory effects of CPA. Histograms show the densitometric quantification of western blots for surface-expressed GluA2 AMPARs. (B) Biotinylation of cultured hippocampal neurons showed a significant decrease in GluA1 surface expression after prolonged CPA treatment. This effect was abolished by the presence of Tat-GluA2-3Y peptide, but not by scrambled Tat-GluA2-3Y peptide. Note the absence of beta actin bands in blots containing the biotinylated GluA2 or GluA1. Bar chart summaries represent biotinylated GluA2 or GluA1 signals normalized to their respective whole hippocampal neuronal lysate signals. Mean±SEM, N=4 from four independent experiments, and *denotes $p<0.05$. C-D. Activation of A1R-induced AMPAR endocytosis is dynamin-dependent. (C) Immunoblots are of streptavidin precipitates probed with mouse anti-GluA2 antibody. Dynasore (100μM), an inhibitor of dynamin GTPase, prevented the CPA-induced GluA2 endocytosis in hippocampal brain slices. GluA2 levels remained constant in whole hippocampal slice lysates. (D) GluA1 surface proteins detected with rabbit anti-GluA1 were also significantly reduced by prolonged CPA application. Dynasore prevented the CPA-induced decrease in GluA1 surface expression. Beta actin was absent in surface biotinylation blots, confirming little or no contamination of biotinylated AMPARs with cytosolic proteins. Biotinylated signals were normalized to GluA2 or GluA1 signals detected in whole hippocampal brain lysates. Mean ± SEM, N=3 from three independent experiments using a specific GluA2 or GluA1 antibody. *denotes $p<0.05$ and **denotes $p<0.01$.

2.3.4 Prolonged A1R stimulation causes A1R-induced persistent synaptic depression (APSD)

Previously, we reported that short-term application of the selective A1R agonist CPA (50nM, 10min) caused significant synaptic depression, however, a persistent synaptic depression of approximately 25% remained after 1h of withdrawal of the agonist and was accompanied by a recovery of paired pulse ratio back to baseline levels (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). In contrast, a 30min application of 500nM CPA produced an even greater APSD (~50-60%, Figure 2.3.4A). Based on our coimmunoprecipitation findings described above, we tested the hypothesis that the biochemical associations of GluA2 and GluA1 with A1Rs could facilitate the expression of persistent synaptic depression, which alterations of AMPAR levels or function. Using the Tat-GluA2-3Y peptide to inhibit GluA2 endocytosis, we found that the APSD levels are lower (~30% *vs.* 55-60%) when slices were pre-incubated with 2μM Tat-GluA2-3Y peptide compared with no peptide treatment or treatment with the scrambled Tat-GluA2-3Y peptide (Figure 2.3.4A, bottom right panel), indicating that clathrin-mediated GluA2 internalization mediates in part the induction of APSD. Interestingly, the Tat-GluA2-3Y peptide also inhibited the short-term synaptic depression during CPA application (~50% vs. ~70%, Figure 2.3.4A bottom left panel), possibly indicating that acute application of the A1R agonist rapidly activated signaling pathways that contributed to GluA2 and GluA1 endocytosis. It is noteworthy that both p38 MAPK and JNK were shown to be maximally activated within the first 10min of CPA application (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007), and both protein kinases have been implicated in glutamate receptor trafficking. The small but significant attenuation of fEPSPs by Tat-GluA2-3Y peptide during a 30min CPA application suggested that the molecular mechanisms of acute CPA-mediated synaptic depression may involve changes at both presynaptic sites (Brust, Cayabyab et al. 2007) as well as postsynaptic sites. Moreover, the Tat-GluA2-3Y peptide significantly attenuated APSD in control or scrambled peptide-incubated hippocampal slices, where synaptic depression decreased by only ~30% in Tat-GluA2-3Y *vs.* 50-60% for control (no peptide) or scrambled Tat-GluA2-3Y groups (Figure 2.3.4A, bottom right panel). This indicated that the molecular mechanisms during APSD (i.e., after ~1h CPA washout) also involve clathrin-mediated GluA2-internalization at a postsynaptic

locus. Consistent with this idea, the paired-pulse facilitation observed during CPA application returned to baseline levels during APSD despite the presence of significant and persistent synaptic depression during extended CPA washout period (Figure 2.3.4B).

In addition, we determined that the APSD levels observed after ~1h washout of CPA was not likely the result of a persistent binding of CPA to A1Rs in hippocampal slices, since a subsequent 30min application of the A1R antagonist DPCPX (500nM) did not modify the levels of APSD. The normalized fEPSP slope values in this control (no peptide) group differed significantly (one-way ANOVA $P<0.0001$, n=7 animals) as follows: control (100±0%), 30min CPA (17±6.1%, $P<0.001$ *vs.* control), 1h CPA washout (48.4±9.4%, $P<0.001$ *vs.* control), and 30min DPCPX (57±5.6%, $P<0.001$ *vs.* control, $P>0.05$ *vs.* 1h CPA washout). These results further indicate that functional interactions between A1Rs and GluA2-containing AMPARs in postsynaptic sites facilitate the clathrin-mediated endocytosis of AMPARs and the induction of APSDs.

Figure 2.3.4

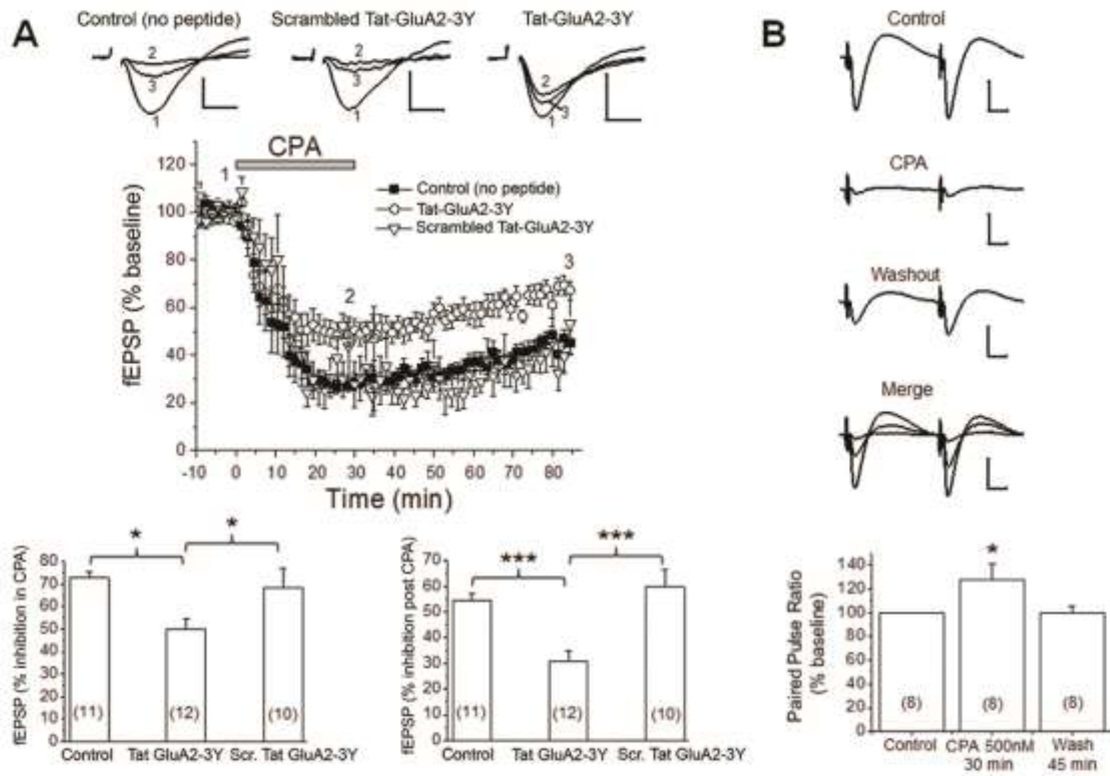


Figure 2.3.4 Prolonged stimulation of A1Rs caused A1R-induced persistent synaptic depression (APSD) in part via clathrin-mediated GluA2 internalization. (A) Top, representative fEPSP traces from hippocampal CA1 region in the absence of Tat-peptides (Control), in scrambled Tat-GluA2-3Y peptide (2μM), and in Tat-GluA2-3Y peptide (2μM). The numbers associated with the fEPSP traces correspond to baseline control (1), 30 min after CPA application (2), and 55 min after CPA washout (3), and also apply to middle panel. The time course of CPA-induced synaptic depression is summarized in the middle panel, showing that Tat-GluA2-3Y peptide, but not its scrambled version, partially inhibited the CPA-induced APSD. Bottom panel, Control (no peptide) and scrambled Tat-GluA2-3Y produced similar levels of synaptic depression during CPA application (left panel) and after CPA washout (right panel), whereas the Tat-GluA2-3Y peptide significantly attenuated these responses (*P<0.05, ***P<0.001 *vs.* Control (no peptide) or scrambled Tat-GluA2-3Y by Student-Neuman-Keuls post hoc test). (B) Paired-pulse stimulation shows that synaptic depression during CPA was accompanied by significant paired pulse facilitation (bottom panel, *P<0.05 vs. Control), but synaptic depression during APSD showed paired pulse ratios similar to baseline control levels. Numbers inside summary bar charts refer to the number of brain slices from different animals. Data are means±SEM. Vertical scale bars are 0.5mV, and horizontal scale bars are 5ms in A and 10ms in B. These results indicate a functional interaction between A1Rs and AMPARs, leading to clathrin-mediated internalization of GluA2-containing AMPARs and subsequent induction of APSDs

2.3.5 Confocal imaging analysis revealed A1R stimulation mediates clathrin-mediated internalization of GluA2 and GluA1 AMPARs in hippocampal neurons

To confirm our biochemical findings that A1R stimulation decreases GluA2 and GluA1 surface levels, we used confocal imaging and antibodies that recognized the extracellular epitopes of GluA2 and GluA1 proteins to quantify the surface expression of GluA2 and GluA1 AMPARs from primary cultured hippocampal neurons. As shown in Figure 2.3.5, stimulation of A1Rs with CPA produced a similar decrease in surface levels of GluA2 (~25% decreased, Figure 2.3.5A-B) and GluA1 (~20% decreased, Figure 2.3.5C-D) expressed on dendritic surfaces located 5μm away from the cell somas. These CPA-induced reductions in surface AMPARs were blocked by either Tat-GluA2-3Y peptide or the A1R antagonist DPCPX, but not by the scrambled Tat-GluA2-3Y peptide (Figure 2.3.5B, D).

These results indicate that functional A1Rs are required for stimulation of A1R-induced GluA2 and GluA1 internalization. Moreover, since the concentration of CPA (500nM) used in this study may very well be causing significant occupation and subsequent activation of A2A receptors, we also determined whether the CPA effects involved functional A2ARs. However, pre-incubation of hippocampal neurons with the A2A receptor antagonist SCH 58261 did not prevent CPA-induced GluA2 and GluA1 internalization, and the A2A receptor agonist stimulation with CGS 21680 did not mimic the inhibitory effect of the A1R agonist CPA on GluA2 and GluA1 surface expression (summarized in Figure 2.3.5B, D). The A2A receptor agonist significantly potentiated surface levels of GluA1 but not GluA2 (Figure 2.3.5D). Together, these findings suggest that GluA2 and GluA1 AMPARs selectively and functionally interact with A1Rs, but not with A2ARs, to promote clathrin-mediated endocytosis of GluA2-containing AMPARs.

Figure 2.3.5

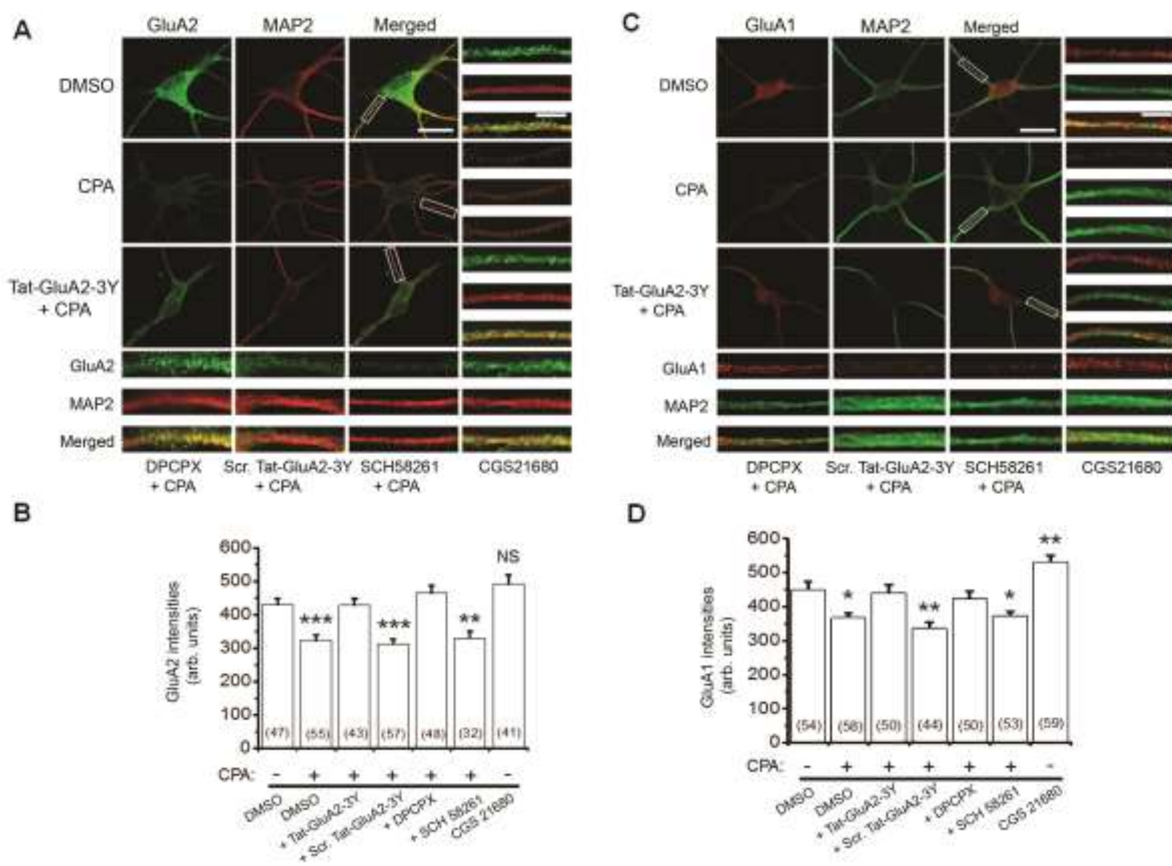


Figure 2.3.5 AMPAR surface levels were decreased by activation of A1Rs with CPA. (A) Confocal imaging of surface GluA2 (green) in primary hippocampal neurons. GluA2 receptors were first labeled without membrane permeabilization and subsequent immuno-labelling of MAP2 was performed after permeabilization with 0.25% Triton X-100. These images show that most dendritic processes when exposed to prolonged A1R agonist CPA (500nM), demonstrate reduced surface GluA2 whereas pre-incubation of hippocampal neurons with Tat-GluA2-3Y (2μM) peptide prevented activation of A1R-induced GluA2 internalization. (B) Summary bar chart showing that activation of A1R-induced GluA2 internalization requires clathrin-mediated endocytosis (shown with Tat-GluA2-3Y) and functional A1Rs (shown with DPCPX (100nM)). The A2A receptor antagonist, SCH 58261 (30nM), did not prevent activation of A1R-induced GluA2 internalization, whereas the A2AR agonist CGS 21680 (10nM) did not mimic the effect of CPA. (C-D) Similar to GluA2, the surface GluA1 levels were decreased by activation of A1R (with CPA). (C) Representative confocal images show that CPA decreased surface levels of GluA1 (red), but not in the presence of Tat-GluA2-3Y peptide or DPCPX. (D) Summary bar chart showing that activation of A1Rs induced surface GluA1 internalization, which was prevented by Tat-GluA2-3Y peptide and DPCPX, but not by scrambled Tat-GluA2-3Y peptide and SCH 58261. However, CGS 21680 significantly increased surface levels of GluA1. Average intensity values in bars represent the mean±SEM and n-values of the number of neurons used are indicated in the bar charts. Statistical significance assessed using one-way ANOVA, followed by post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0001, NS p>0.05.

2.3.6 GluA2-containing AMPARs are regulated by A1R-mediated activation of p38 MAPK, JNK and PP2A in hippocampal brain slices

In previous studies, we showed that activation of A1Rs by CPA leads to increased activity of p38 MAPK and JNK, and that A1Rs and the p38 MAPK were found in the same protein complex (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). We hypothesized that p38 MAPK and JNK activation converged on signaling pathway(s) activated by A1Rs and cause internalization of GluA2-containing AMPARs. To determine whether A1R-p38 MAPK and A1R-JNK signaling pathways are involved in A1R-induced internalization of GluA2, rat hippocampal slices were pre-incubated with the p38 MAPK inhibitor SB203580 (20μM) alone or in combination with the JNK inhibitor II (5μM) for 1h before CPA applications. After separating the membrane from cytosolic fractions, immunoblotting was performed to quantify the levels of GluA2 in hippocampal membrane fractions. We found that the p38 MAPK inhibitor fully inhibited the CPA-induced attenuation of GluA2 levels in membrane fractions (Figure 2.3.6A). CPA treatment alone caused a ~48% decrease in membrane GluA2 levels. However, pre-incubation of hippocampal slices with both p38 MAPK and JNK inhibitors not only prevented the A1R-induced decrease in GluA2 membrane levels, this drug combination also significantly increased the GluA2 membrane levels by 3-fold (Figure 2.3.6A, bottom panel).

To confirm these results obtained from membrane fractions and to begin to address potential side effects of drug inhibitors, we also pre-incubated hippocampal slices with SB203580 (20μM, p38 MAPK inhibitor), SB202474 (20μM, Negative control of SB203580), JNK II inhibitor (5μM) or JNK II negative inhibitor (5μM) prior to CPA incubation (500nM, 45min). The surface proteins were isolated using biotinylation and quantified by western blotting. Incubation of the slices with either SB203580 or JNK II inhibitor prevented A1R-induced internalization of GluA2-containing AMPARs, whereas their respective inactive analogs were ineffective in blocking the GluA2 internalization (Figure 2.3.6B).

Phosphorylation of AMPARs is important for trafficking of AMPARs (Shepherd and Huganir 2007). Activation of phosphorylated p38 MAPK by A1 receptor stimulation

Figure 2.3.6

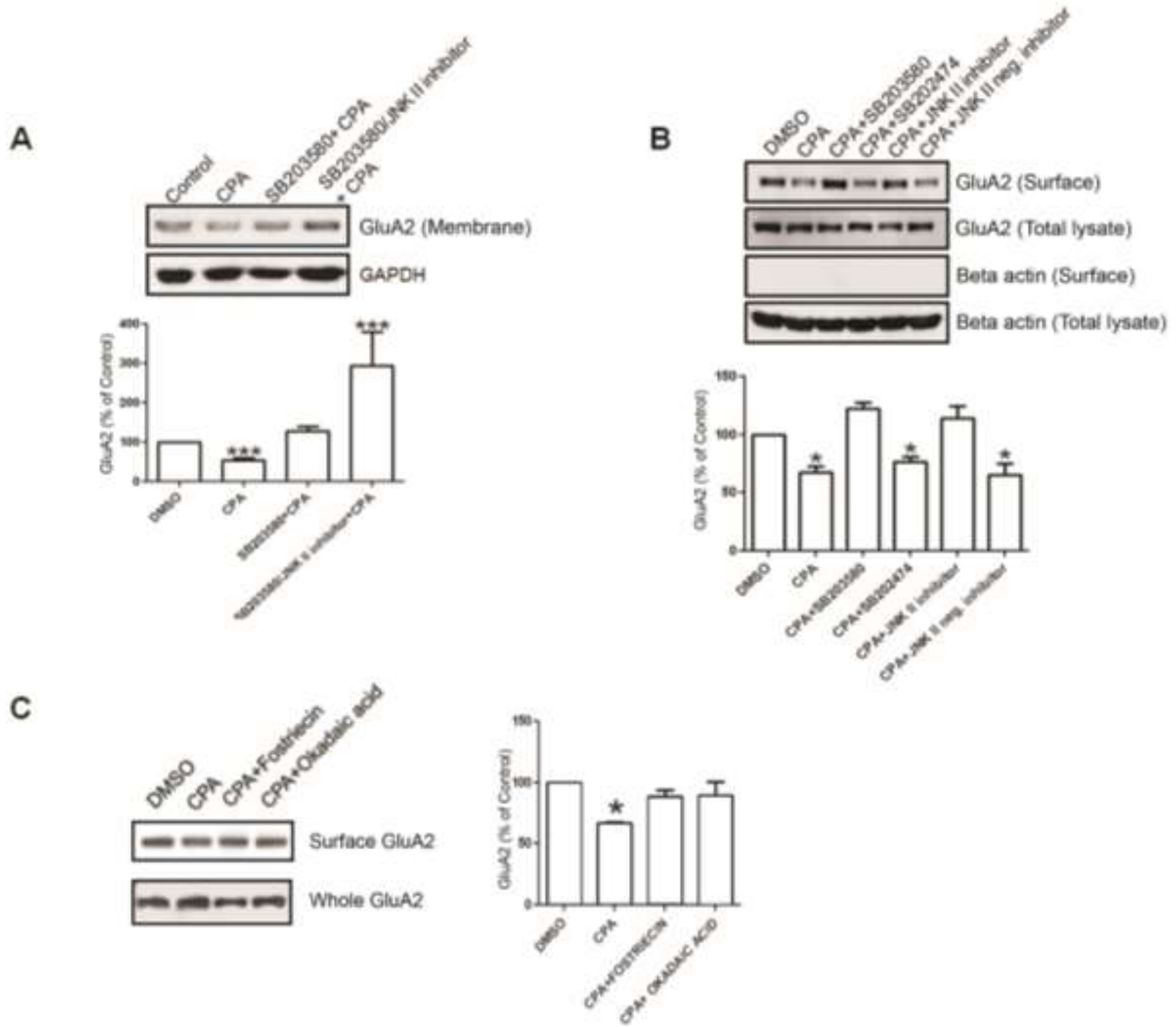


Figure 2.3.6 A1R-mediated internalization of GluA2-containing AMPARs in hippocampal slices is differentially regulated by p38 MAPK, JNK, and PP2A. (A) Levels of GluA2 (top) and GAPDH (bottom) in the membrane fraction with the indicated treatments. The summary bar chart shows that activation of A1Rs by CPA (500nM, 45min) produced a 48% decrease in GluA2 membrane expression. Pre-incubation with a p38 MAPK inhibitor (SB203580, 20μM) prior to CPA application (500nM, 45min) significantly reduced GluA2 internalization. Pre-incubation with both p38 MAPK and JNK II (5μM) inhibitors produced GluA2 levels above N-values are as follows: for Control (n=8 independent blots), CPA (n=8), SB203580 (n=5), and SB203580 + JNK inhibitor II (n=7). (B) In surface biotinylation studies, p38 MAPK inhibitor SB203580 (20μM) but not the inactive analog SB202474 (20μM) and JNK II inhibitor (5μM), but not its inactive analog JNK II negative control (5μM) individually prevented CPA-mediated GluA2 internalization. Hippocampal slices were pre-incubated with SB203580, SB202474 (20μM), JNK II inhibitor and JNK II negative inhibitor (5μM) for 1h prior to CPA treatment (500nM,45 min). (C) Surface biotinylation study of hippocampal slices pre-incubated for 1h prior to CPA treatment (500nM, 45min) in DMSO (control), or one of the PP2A inhibitors Okadaic Acid (20nM), or Fostriecin (20nM). Surface levels of GluA2 after CPA treatment were significantly reduced, as shown before, and this surface reduction was prevented by treatment with PP2A inhibitor treatment. Intensity values in summary bar chart represent the mean±SEM from n=4 independent experiments; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

induced translocation of PP2A (protein phosphatase 2A) to the cell membrane (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). Therefore, modulation of AMPARs in the brain by phosphorylation may play a role in APSD. To determine whether PP2A is involved in A1R-induced internalization of GluA2, rat hippocampal slices were pre-incubated with the PP2A inhibitor Fostriecin (20nM) or Okadaic acid (20nM) for 1h before CPA applications (500nM, 45min). Both PP2A inhibitors prevented the internalization of GluA2-containing AMPARs induced by CPA (Figure 2.3.6C), suggesting PP2A is involved in A1R-induced GluA2 AMPARs internalization. Together, these data indicate that p38 MAPK, JNK and PP2A are involved in clathrin-mediated endocytosis of GluA2 AMPARs.

2.3.7 GluA1-containing AMPARs are regulated by A1R-mediated activation of PP2A, but not p38 MAPK and JNK in hippocampal brain slices

To determine whether p38 MAPK, JNK and PP2A are involved in A1R-induced GluA1 internalization, rat hippocampal slices were pre-incubated with the p38 MAPK inhibitor SB203580 (20μM) alone or in combination with the JNK inhibitor II (5μM) for 1h before CPA application (500nM, 45min). After separating the membrane from cytosolic fractions, immunoblotting was performed to quantify the levels of GluA1 in hippocampal membrane fractions. Pre-incubation of p38 MAPK and JNK inhibitors did not inhibit the CPA-induced attenuation of GluA1 levels in membrane fractions (Figure 2.3.7A, summarized in bottom panel).

Biotinylation experiments were also performed to confirm the results obtained from membrane fractions. We pre-incubated hippocampal slices with SB203580 (20μM), SB202474 (20μM), JNK II inhibitor (5μM) or JNK II negative inhibitor (5μM) prior to CPA incubation (500nM, 45min). Incubation of the slices with either SB203580 or JNK II inhibitor did not prevented the A1R-induced internalization of GluA1 AMPARs. Their respective inactive analogs were also ineffective in blocking the GluA1 internalization (Figure 2.3.7B). Similar to the results obtained from hippocampal membrane fractions

Figure 2.3.7

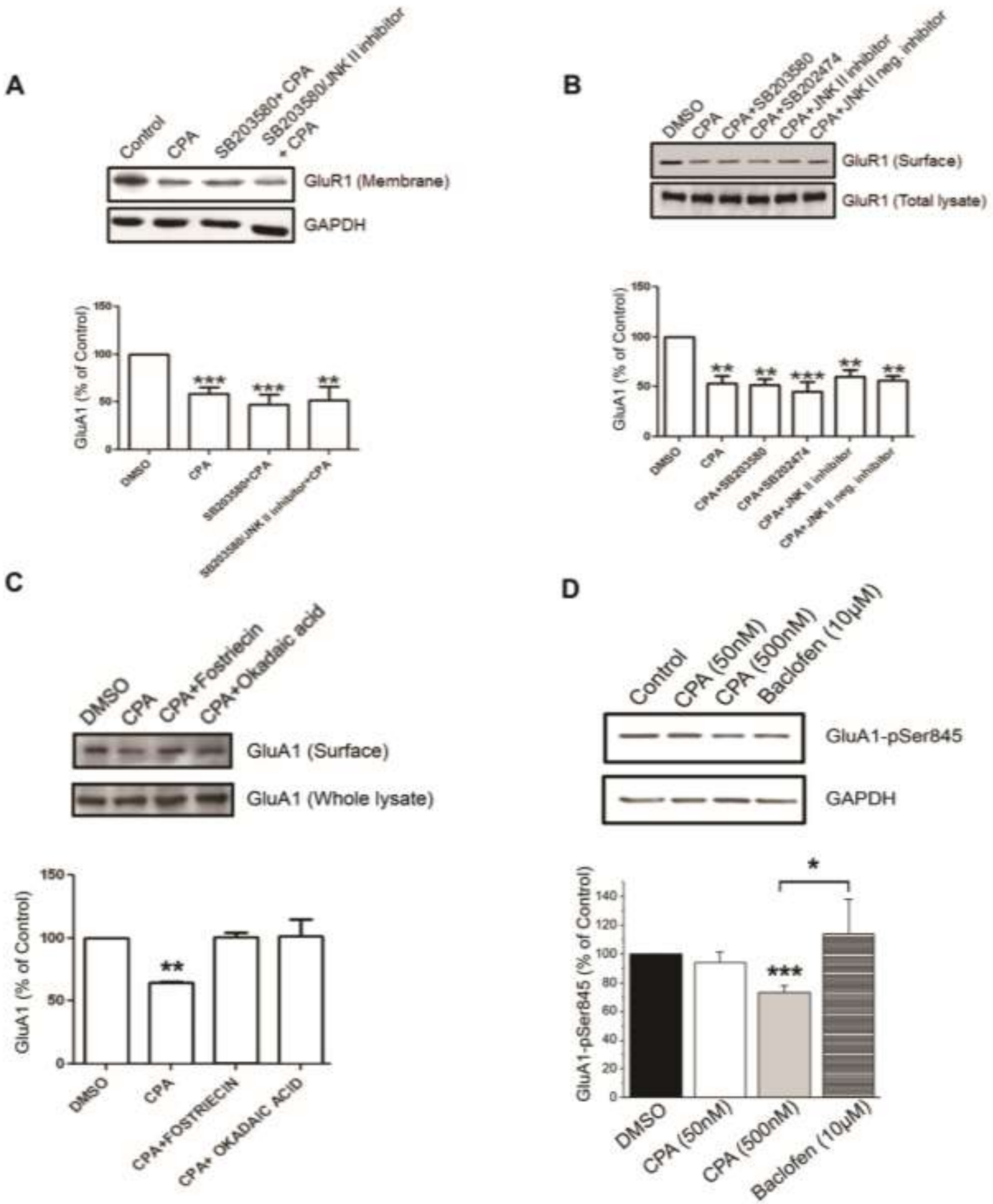


Figure 2.3.7 A1R-mediated internalization of GluA1-containing AMPARs in hippocampal slices is not regulated by p38 MAPK or JNK, but is regulated by PP2A, and robust A1R activation reduced GluA1-Ser845 phosphorylation. (A) Levels of GluA1 (top) and GAPDH (bottom) in the membrane fraction of hippocampal slice lysates with pre-incubation in DMSO (control), SB203580 (20μM), or SB203580 and JNK II inhibitor (5μM) together followed by CPA treatment (500nM, 45min). Both p38 MAPK inhibitor SB203580 alone and SB203580 plus JNK II inhibitor did not prevent A1R-induced GluA1 internalization. (B) Surface biotinylation of hippocampal slices showing that A1R-mediated GluA1 internalization did not depend on the activity of p38 MAPK and JNK. Compared to GluA2, Drug treatments with SB203580 (20μM), but not the inactive analog SB202474 (20μM), or JNK II inhibitor (5μM), or the inactive analogs of SB203580 (SB202474, 20μM) or JNK II inhibitor (JNK II neg. inhibitor, 5μM), did not prevent A1R-induced internalization of GluA1. (C) Pre-incubation of hippocampal slices in the PP2A inhibitors Okadaic Acid (20nM) or Fostriecin (20nM) for 1h followed by CPA treatment (500nM, 45min) prevented A1R-induced internalization of GluA1. (D) Whole lysates of hippocampal slices treated with CPA (50nM or 500nM, 45min) or the $GABA_B$ receptor antagonist Baclofen (10μM, 45min) and probed for the C-terminal phosphorylation site GluA1-pSer845. The antibody used was specific for phosphorylated Ser845 (pSer845) of GluA1. CPA treatment of 500nM caused a robust decrease in pSer845, whereas Baclofen and 50nM CPA did not. All values in summary bar charts are means±SEM, with * $p<0.05$, ** $p<0.01$; *** $p<0.001$.

(Figure 2.3.7A), A1R-mediated GluA1 internalization was also unaltered by active or inactive analogs of p38 MAPK and JNK inhibitors (Figure 2.3.7B).

To determine whether PP2A is involved in the A1R-induced GluA1 internalization, hippocampal slices were pre-incubated with the PP2A inhibitors Fostriecin (20nM) or Okadaic Acid (20nM) for 1h before CPA applications. The PP2A inhibitors prevented the internalization of GluA1 induced by CPA alone (Figure 2.3.7C), suggesting PP2A is involved in A1R-induced GluA1 internalization. Because CPA induces PP2A translocation to hippocampal membrane fraction (Brust, Cayabyab et al. 2006), and PP2A can dephosphorylate GluA1 at Serine845 (Ser845) (Snyder, Allen et al. 2000), we predicted that CPA application decreases the phosphorylation of GluA1 at Serine 845. The resultant blots showed that incubation of CPA (500nM), but not the low CPA concentration (50nM) or Baclofen (10μM), which is an agonist for the $GABA_B$ receptors (Mezler, Muller et al. 2001), Resultant blots showed decreased phosphorylation of GluA1 at Ser845 (Figure 2.3.7D). The results suggest that dephosphorylation levels of GluA1 at Ser845 specifically are induced by the activation of A1R but not activation of $GABA_B$. The results also show that the activation of A1Rs by 500nM CPA, but not 50nM CPA, induced robust reductions of GluA1-pSer845. Together, these data indicate that PP2A, but not p38 MAPK and JNK, is involved in clathrin-mediated endocytosis of GluA1 AMPARs, and that the internalization of GluA1 is correlated with a reduction of phosphorylated GluA1 at Ser845.

2.3.8 Selective inhibition of p38 MAPK and JNK by shRNA transfections prevented A1R-mediated GluA2 internalization in cultured hippocampal neurons

To further address the dependence of GluA2-containing AMPAR internalization on A1R-mediated p38 MAPK and JNK activation, we used confocal imaging of cultured hippocampal neurons and compared the effects of pharmacological inhibitors and genetic knockdown of p38 MAPK and JNK. Cultured hippocampal neurons were pretreated with the p38 MAPK inhibitor SB203580, SB202474 (inactive p38 MAPK inhibitor), JNK II inhibitor or JNK II negative inhibitor (concentrations same as above) and then stimulated with 500nM CPA for 45min. As shown in Figure 2.3.8A, surface levels of GluA2 were

significantly decreased by CPA, but not in the presence of SB203580 or JNK II inhibitor. Indeed, the JNK II inhibitor not only blunted CPA-induced GluA2 internalization, it also potentiated GluA2 surface levels (Figure 2.3.8A, right panel). In contrast, the respective negative control compounds SB202474 (for p38 MAPK) or the JNK II negative inhibitor (for JNK) did not prevent CPA-induced GluA2 internalization.

We also tested the effects of p38 MAPK and JNK inhibitors on A1R-mediated GluA1 internalization, and found that these pharmacological inhibitors (SB203580 and JNK II inhibitor) did not significantly alter the levels of surface-expressed GluA1, using an antibody directed against an extracellular epitope in non-permeabilized condition. The GluA1 intensity values (arbitrary units) obtained using a similar analysis performed for GluA2 were as follows: Control GluA1 (691.5±55.4, n=36), CPA (310.6±24.9, n=15, P<0.001 *vs.* control), SB203580 + CPA (433.1±63.4, n=16, P<0.05 *vs.* control), SB202474 + CPA (287.6±44.0, n=22, P<0.001 *vs.* control), JNK II inhibitor + CPA (503.9±39.5, n=33, P<0.01 *vs.* control), and JNK II negative control + CPA (468.1±29.6, n=41, P<001 *vs.* control). These imaging results are in agreement with our biochemical studies (Figure 2.3.6 and 2.3.7), indicating that GluA2 and GluA1 internalizations are differentially regulated by A1R-mediated p38 MAPK and JNK activation.

In our previous report (Brust, Cayabyab et al. 2007), we found that both SB203580 and JNK II inhibitor (also called SP600125) inhibited A1R-mediated phospho-JNK2/3 elevation, raising the possibility that these drugs may have narrow specificity for p38 MAPK and JNK or that JNK activation is dependent on p38 MAPK activation. We therefore compared the effects of pharmacological inhibitors of p38 MAPK and JNK on CPA-mediated GluA2 internalization with those effects using genetic knockdown of p38 MAPK and JNK. We transfected hippocampal neurons with p38α MAPK shRNA, JNK1 shRNA or the control shRNA Plasmid A separately. Using western blotting, we confirmed that two days after transfection the expression level of p38α MAPK was decreased by 45% and the level of JNK1 was decreased by 46% compared to control

Figure 2.3.8

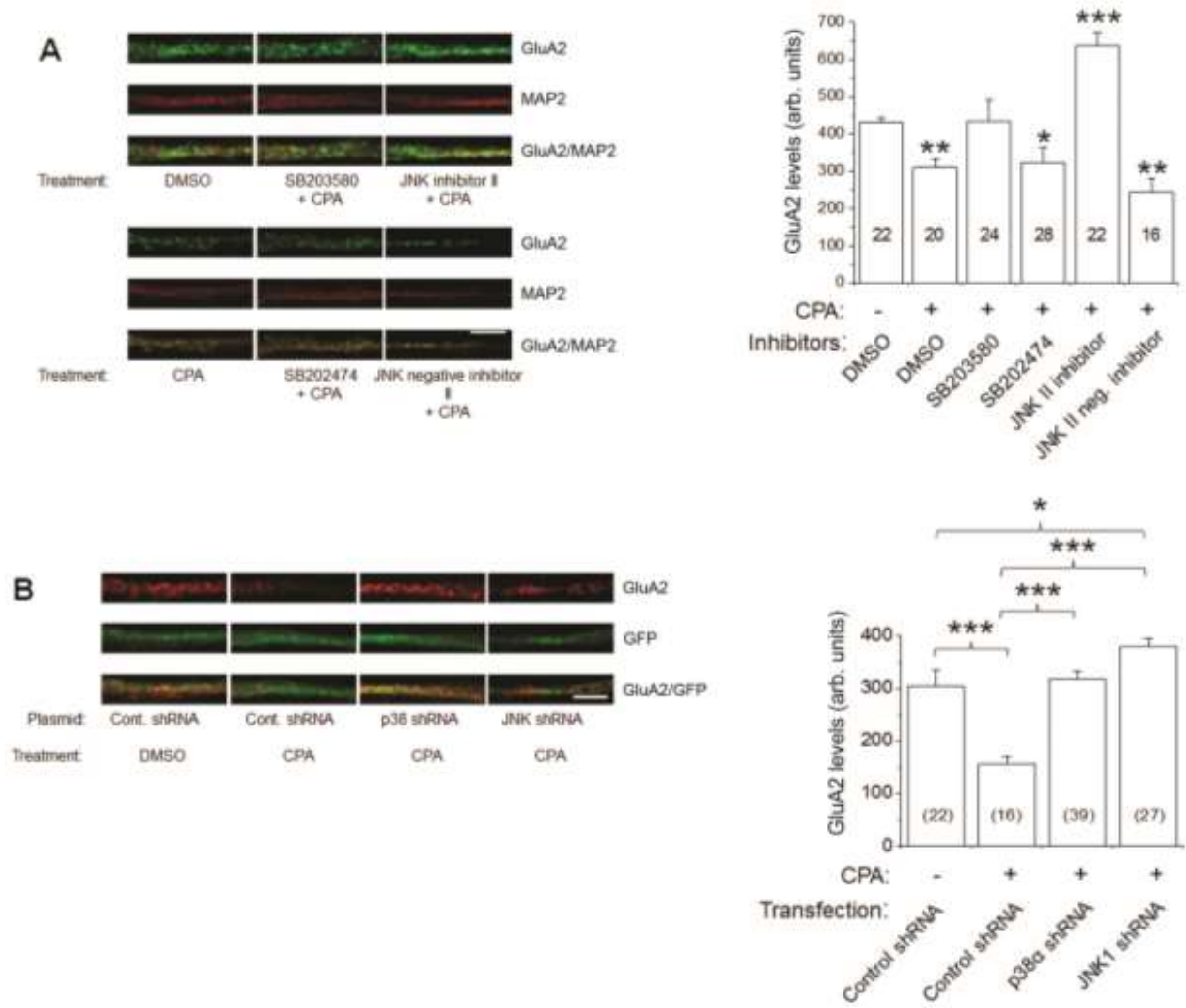


Figure 2.3.8 A1R-mediated decrease in GluA2 surface levels in primary hippocampal neurons was prevented by pharmacological inhibitors and genetic knockdown of p38 MAPK and JNK. (A) Intensity levels of surface-expressed GluA2 were determined by confocal imaging and analyzing 10μm dendritic lengths located 5μm away from cell somas. Results showed A1R-induced GluA2 endocytosis was inhibited by p38 MAPK inhibitor SB203580 (20μM), but not by inactive analog SB202474, and JNK II inhibitor (5μM), but not by its inactive analog JNK II negative control. The compounds SB203580, SB202474, JNK II inhibitor and JNK II negative inhibitor were applied to hippocampal neurons for 1h prior to CPA treatment (500nM, 45 min). Surface GluA2 (green) was detected by using an antibody directed against the extracellular epitope of GluA2 in non-permeabilized conditions, then subsequently permeabilized and stained with chicken anti-MAP2 antibody (red). The p38 MAPK and JNK inhibitors did not significantly affect CPA-mediated GluA1 internalization (see text in Results for values) (B) Using a shRNA knockdown strategy, the shRNAs p38α MAPK and JNK1 prevented A1R-induced GluA2 internalization. Cultured neurons transfected with the control plasmid A (GFP-fluorescent), p38α MAPK, or JNK1 shRNA were treated with DMSO or CPA (500nM, 45min) and subsequently labeled with GluA2 and MAP2 as in (A) CPA-induced GluA2 internalization was prevented by transfections of p38α MAPK and JNK1 shRNA plasmids. Average GluA2 intensity values in summary bar charts represent the mean±SEM from 3 transfections, with the number of neurons indicated inside brackets, and where $^{*}p<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* control. Statistical significance was assessed using one-way ANOVA, followed by post hoc Student Newman-Keuls test. Calibration is 2μm, and applies to panels in A and B.

shRNA transfections (data not shown). Confocal imaging analysis revealed that two days after transfection of hippocampal neurons with control Plasmid A, p38 MAPK shRNA or JNK shRNA and subsequent A1R stimulation with CPA (500nM for 45min), the A1R-induced GluA2 internalization was completely abrogated by the p38α MAPK or JNK1 shRNAs (Figure 2.3.8B). A modest but significant increase in GluA2 surface levels was also observed in neurons transfected with JNK1 shRNA (Figure 2.3.8B, right panel). Together, these results indicate that stimulation of A1R-induced GluA2 internalization is dependent on p38 MAPK and JNK activities.

2.3.9 The A2AR is not involved in GluA2 trafficking, but is involved in GluA1 trafficking

As per the coimmunoprecipitation and colocalization results shown in Figures 2.3.1 and 2.3.2, A2ARs are not physically associated with GluA1 and GluA2 AMPARs. However, this does not preclude a functional interaction between A2ARs and AMPARs. Indeed, it has been shown that selective agonist activation of A2ARs increased the GluA1 level in hippocampus (Dias, Ribeiro et al. 2010). To determine whether the stimulation of the A2ARs alter GluA2 surface expression, we incubated hippocampal slices with the A2AR agonist CGS 21680 (10nM), or the A2AR antagonist SCH 58261 (30nM) for 1h. The results showed that CGS 21680 (Figure 2.3.9A) and SCH 58261 (Figure 2.3.9B) did not alter the surface level of GluA2, suggesting the stimulation of A2ARs does not change the surface level of GluA2. To test whether the stimulation of A2ARs alter the surface expression of GluA1, we also quantified the GluA1 surface levels with treatment of CGS 21680 or SCH 58261 in hippocampal slices. The results showed that CGS 21680 (Figure 2.3.9A), but not SCH 58261 (Figure 2.3.9B), increased the surface level of GluA1, indicating that the stimulation of A2ARs increase the surface level of GluA1.

To determine whether A1R stimulation and A2AR stimulation alter surface expression of either adenosine receptor, we pre-incubated hippocampal slices with CGS 21680 (10nM) alone or CGS 21680 (10nM) for 1h prior to CPA incubation (500nM, 45 min). Incubation of the slices with CGS 21680 (Figure 2.3.9C) or SCH 58261 (Figure 2.3.9D) did not prevent the CPA-induced internalization of A1R. However, SCH 58261

Figure 2.3.9

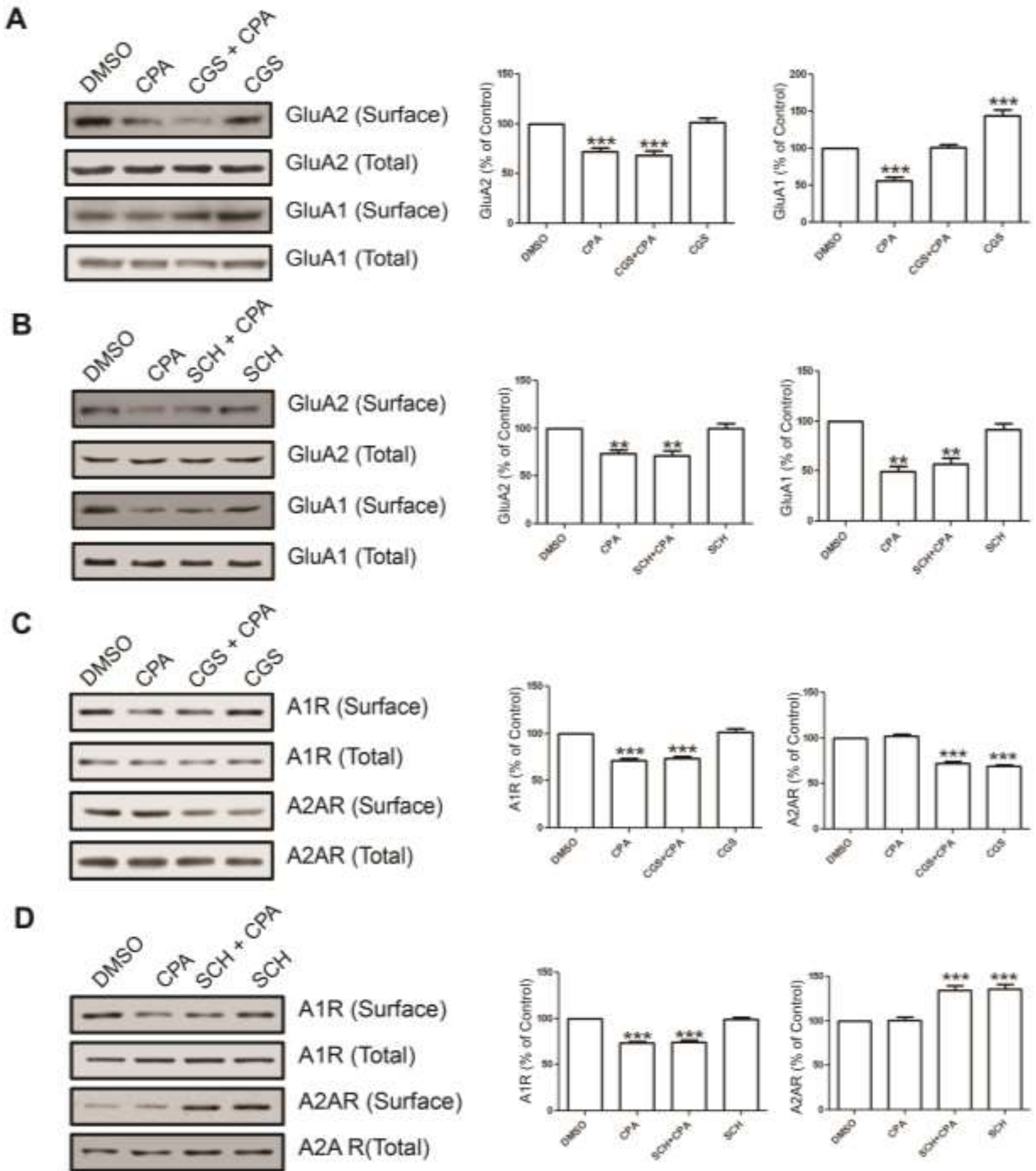


Figure 2.3.9 The A2AR does not affect A1R-induced GluA2 internalization, but does affect A1R-induced GluA1 internalization. (A) Surface biotinylation of hippocampal slices that were pre-incubated with DMSO (control) or CGS 21680 (10nM), an A2AR agonist, for 1h followed by CPA treatment (500nM, 45min). Blots (left) were probed for GluA2 and GluA1 levels and summary bar charts (right) show that CGS 21680 did not affect either GluA2 surface expression with CGS 21680 alone or in CGS 21680 with CPA treatment (left bar chart). Conversely, CGS 21680 prevented CPA-induced GluA1 internalization, and CGS 21680 by itself significantly increased GluA1 surface levels without CPA treatment (right bar chart). (B) Biotinylation of hippocampal slices pre-incubated in SCH 58261 (30nM), an A2AR antagonist followed by CPA treatment (500nM, 45min). Summary bar charts show that SCH 58261 did not significantly affect CPA's effect on the surface levels of GluA2 and GluA1. (C) Hippocampal biotinylation using the same protocol as in (A) labeled for A1R and A2AR. Summary char chart for A1R (left chart) shows that CPA and CGS 21680 with CPA-induced a reduction in surface A1Rs, and were not affected by CGS 21680 by itself. The A2AR surface levels (right chart) were not affected by CPA treatment alone, but were reduced with CGS 21680 treatments. (D) Using the same drug treatments as in (B), biotinylation shows A1R and A2AR expression levels with CPA, SCH 58261, or CPA and SCH 58261 together. Summary bar chart for A1R (left chart) shows that CPA alone and CPA with SCH 58261 induced reduced surface levels of A1R, but SCH 58261 alone did not affect A1R levels. A2AR surface levels (right chart) show that CPA and SCH 58261 together as well as SCH58261 by itself caused an increase in A2AR surface levels, but CPA alone did not affect surface levels. All values are means±SEM, with $^{**}p<0.01$; $^{***}p<0.001$.

alone or in combination with CPA increased A2AR surface levels (Figure 2.3.9D), suggesting that endogenous adenosine tone was sufficient to cause A2AR desensitization in the hippocampal slices. To address the relationship of stimulation and internalization between A1Rs and A2ARs, we incubated the hippocampal slices with the agonists and antagonists of A1Rs and A2ARs. The results show that CPA application alone decreased the surface level of A1Rs, but not A2ARs (Figure 2.3.9C), suggesting CPA specifically stimulates A1Rs. Application of CGS 21680 decreased the surface level of A2ARs, but not A1Rs (Figure 2.3.9C). Pre-incubation of CGS 21680 prior CPA treatment did not prevent CPA-induced A1R internalization, but still caused A2AR internalization (Figure 2.3.9C), suggesting CGS 21680 specifically activates A2ARs. SCH 58261 treatment increased the surface level of A2ARs, but not A1Rs (Figure 2.3.9D) suggesting that SCH 58261 specifically binds A2ARs. Pre-incubation of SCH 58261 prior to CPA did not prevent CPA-induced A1R internalization, suggesting that stimulation of A2ARs is not involved in the CPA-induced A1R internalization. Pre-incubation of SCH 58261 prior to CPA treatment still increased the surface level of A2ARs (Figure 2.3.9D), suggesting A1R stimulation is not involved in SCH 58261-induced increase in surface level of A2ARs. In summary, the A2AR is not involved in A1R-induced internalization of AMPARs, but A2AR stimulation affects the surface level of GluA1, but not GluA2. In addition, stimulation of A1Rs and A2ARs independently alter their surface expression levels.

2.3.10 Hypoxia mediates GluA2 and GluA1 internalization via clathrin-mediated endocytosis

It is widely accepted that hypoxia increases the extracellular levels of adenosine (Van Wylen, Park et al. 1986; Phillis, Walter et al. 1987; Fowler 1993; Dale, Pearson et al. 2000). Due to high concentrations of adenosine in hypoxia, A1Rs are expected to be activated to mediate hypoxia-induced synaptic depression (Fowler 1989). We also previously reported that a 5min hypoxic insult caused significant synaptic depression in CA1 region of hippocampus, and this was shown to be dependent on A1R-mediated

activation of p38 MAPK and JNK (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). Earlier studies by Sebastiao and colleagues provided the first report of an incomplete recovery of synaptic transmission after slightly more prolonged hypoxic insult to hippocampal slices (Lucchi, Latini et al. 1996). In the present study, we performed surface biotinylation and membrane fractionation studies using hippocampal brain slices after a 20min hypoxic insult to test whether prolonged hypoxic insult induces clathrin-mediated internalization of GluA2- and GluA1-containing AMPARs through A1R activation. Hippocampal slices were pre-incubated with Tat-GluA2-3Y or scrambled Tat-peptide for 1h before applying hypoxic stimulation for 20min. After hypoxia, the membrane fractions or the biotinylated proteins were isolated and analyzed by western blotting. As shown in Figure 2.3.10, the hypoxic insult mimicked the effect of selective A1R stimulation with CPA (see Figure 2.3.3), by significantly decreased GluA2 and GluA1 levels in hippocampal membrane fractions (Figure 2.3.10A-B) and in surface biotinylated samples (Figure 2.3.10C-D).

However, the Tat-GluA2-3Y peptide, but not the scrambled Tat-peptide, was also effective in blocking hypoxia-mediated GluA2 and GluA1 internalization, as shown in hippocampal membrane fractions (Figure 2.3.10A-B) and in biotinylated hippocampal tissue (Figure 2.3.10C-D). To confirm that the hypoxia-induced reduction in GluA2 and GluA1 surface expression was caused by A1R stimulation, pre-incubation of hippocampal slices with the A1R antagonist DPCPX blocked these changes in GluA2 and GluA1 surface levels (Figure 2.3.10C-D, summary bar chart). These results indicate a previously unknown mechanism involving excess elevation of adenosine during hypoxia that leads to clathrin-mediated AMPAR internalization and hypoxia-mediated synaptic depression.

Figure 2.3.10

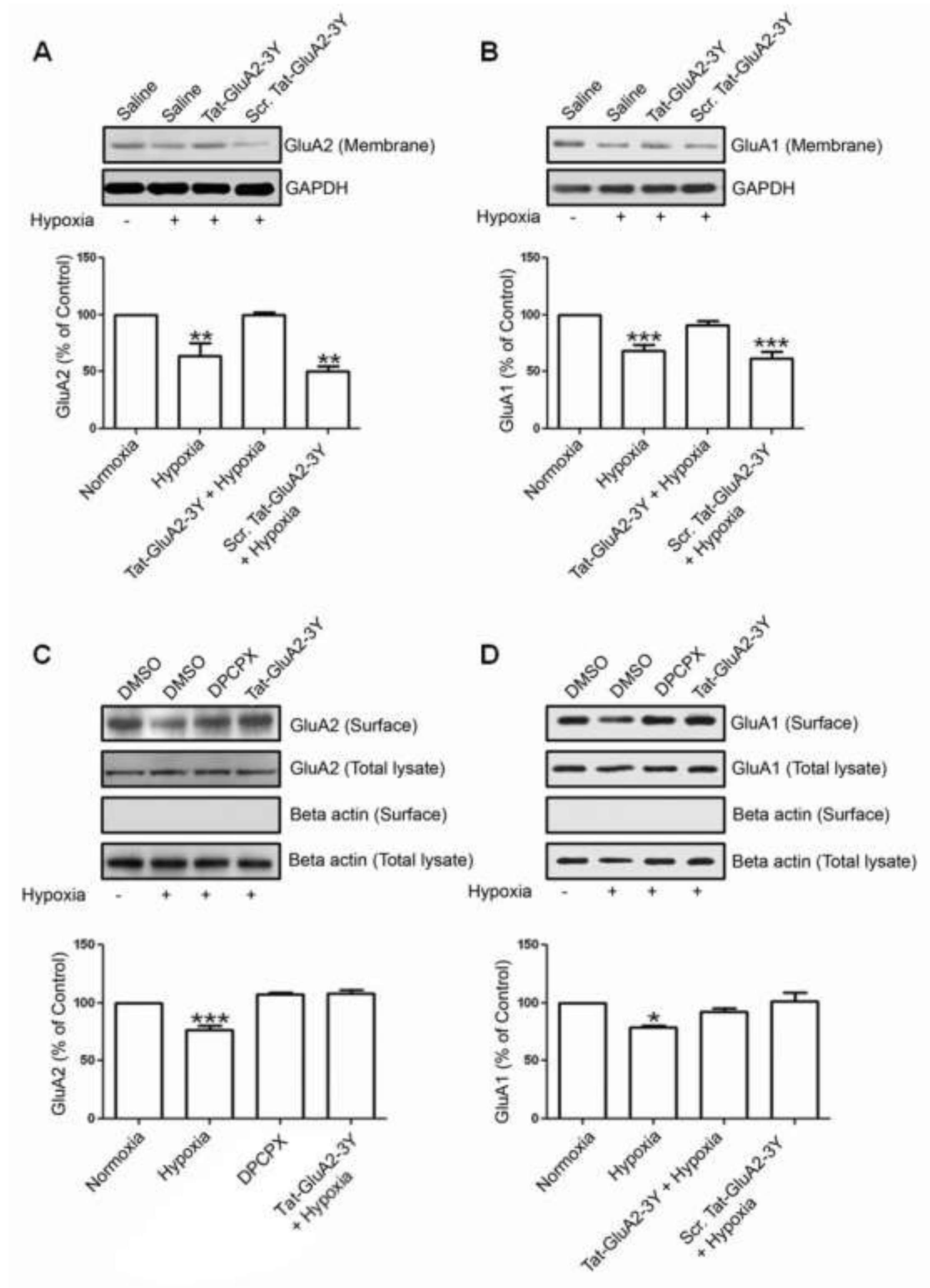


Figure 2.3.10 Hypoxia-induced internalization of both GluA2 and GluA1 requires clathrin-mediated endocytosis and functional A1Rs in rat hippocampus. (A). Hippocampal slices were pre-incubated with Tat-GluA2-3Y (2μM) or scrambled Tat-GluA2-3Y (2μM) peptides for 1h before applying hypoxic insult (ACSF solution saturated with 95% N_2/5% CO_2). After a 20min hypoxic stimulation, hippocampal membrane fractions were isolated and GluA2 levels were subsequently determined by western blotting. GluA2 signals in membrane fractions were normalized to GAPDH values. During hypoxia, membrane expression of GluA2 was significantly decreased, and Tat-GluA2-3Y peptide, but not its scrambled version, prevented this GluA2 downregulation. (B). Similar to GluA2, the Tat-GluA2-3Y peptide prevented the decrease in GluA1 expression in hippocampal membrane fractions. Values in A-B are mean±SEM for GluA2 and GluA1 from six independent experiments, with *$P<0.05$, **$P<0.01$, and ***$P<0.001$ *vs.* control. (C-D) Rat hippocampal slices pre-incubated with DPCPX (500nM) or Tat-GluA2-3Y peptide (2μM) were exposed to a 20min hypoxic insult, and surface proteins were subsequently biotinylated and analyzed by western blotting. Hypoxia-induced decrease in surface GluA2 (C) and GluA1 (D) was prevented by DPCPX and Tat-GluA2-3Y peptide. In contrast, GluA2 and GluA1 levels in whole hippocampal lysates were not altered (second row of blots in panels C-D), and beta actin was only detected in whole lysate blots (fourth row), but not in the biotinylated blots (third row). Values in C-D are mean±SEM from three independent experiments, with *$P<0.05$ and ***$P<0.001$.

2.3.11 Focal cortical ischemia in an *in vivo* PVD small-vessel stroke model alters hippocampal surface expression of AMPARs and adenosine receptors, contributes to tonic synaptic depression, and increases neurodegeneration in the hippocampus

Many focal cerebral ischemia models involve occlusion of large cerebral blood vessels such as the middle cerebral artery, which results in damage to the striatum and cortex to varying degrees depending on the duration of vessel occlusion (Traystman 2003). During hypoxia, transient global ischemia or focal cerebral ischemia, it is well accepted that there is an increase in the extracellular levels of adenosine (Van Wylen, Park et al. 1986; Rudolphi, Schubert et al. 1992; Valtysson, Persson et al. 1998; Dale, Pearson et al. 2000; Chu, Xiong et al. 2013). Brain damage in global and focal ischemia models occurs within selectively vulnerable areas such as the hippocampal CA1 region, neocortex, and striatum (Kirino 1982; Smith, Bendek et al. 1984; McBean and Kelly 1998; Traystman 2003; Prosser-Loose, Verge et al. 2010). Global ischemia has been shown to selectively reduce the expression of GluA2-containing AMPARs in the CA1 region in rats and gerbils (Pellegrini-Giampietro, Zukin et al. 1992; Pollard, Heron et al. 1993; Gorter, Petrozzino et al. 1997; Pellegrini-Giampietro, Gorter et al. 1997). In this study, we have used a modified pial vessel disruption (PVD) protocol, which mimics mild, small-vessel strokes. This involves disruption of class II size pial vessels, and has been shown to produce a consistent cone-shaped cortical lesion damage that does not extend to the corpus callosum (Wang and Walz 2003; Hua and Walz 2006; Cayabyab, Gowribai et al. 2013). Since this represents a permanent non-reperfusion injury model, we hypothesized that adenosine surges will be sufficiently prolonged to cause GluA2 and GluA1 downregulation and induce damage in brain regions distant from the site of injury, such as the hippocampus.

As shown in Figure 2.3.11A-B, GluA2 and GluA1 surface expression in the ipsilateral side of the hippocampus were reduced 2 days after performing the PVD lesion surgeries. Surprisingly, these levels were also downregulated in the contralateral side of the hippocampus. Consistent with our results showing that surface levels of GluA2 and GluA1 are reduced by the A1R agonist CPA, and both AMPAR subunits coimmunoprecipitated with A1Rs, we found that A1R surface expression was reduced both in ipsilateral and

contralateral sides of the lesion (Figure 2.3.11C). This result agrees with previous findings (Coelho, Rebola et al. 2006) showing that hypoxia-mediated increase in extracellular adenosine downregulates A1Rs. In contrast, A2AR surface expression increased in the ipsilateral and contralateral side of the cortical lesion damage. Since it is widely accepted that adenosine is tonically elevated during cerebral ischemia, we tested the hypothesis that adenosine surges in the brain after focal disruption of cortical pial vessels (no reperfusion) may be sufficient to affect vulnerable brain regions, such as the hippocampus, and influence the induction of synaptic depression. Therefore, we evaluated the effects of PVD vs. sham surgeries on synaptic transmission two days post surgeries. The fEPSP recordings from hippocampal slices were obtained from the ipsilateral side of PVD surgery lesion or sham surgery. Consistent with a downregulation of A1Rs after PVD, we observed less synaptic potentiation and paired-pulse depression when the A1R antagonist DPCPX was applied to the PVD slices compared to sham brains (Figure 2.3.12A-B). These data indicate that persistent synaptic depression in PVD hippocampal slices reflects changes in both presynaptic (decreased probability of transmitter release) and postsynaptic (altered levels of AMPARs and adenosine receptors) loci. These results also indicate that a focal cortical ischemia can potentially affect vulnerable areas of the brain distant from the site of injury.

Finally, to quantify neurodegenerative processes in the hippocampus in PVD vs. sham, post-stroke (48h) hippocampal slices were obtained to perform Fluoro-Jade B staining. Confocal imaging results of Fluoro-Jade B staining show that more neurodegeneration was observed in the hippocampus in PVD brain slices compared to sham animals (Figure 2.3.12C). This suggests that the disruption of class II size pial vessels in PVD cause the impairment of hippocampal neurons.

Figure 2.3.11

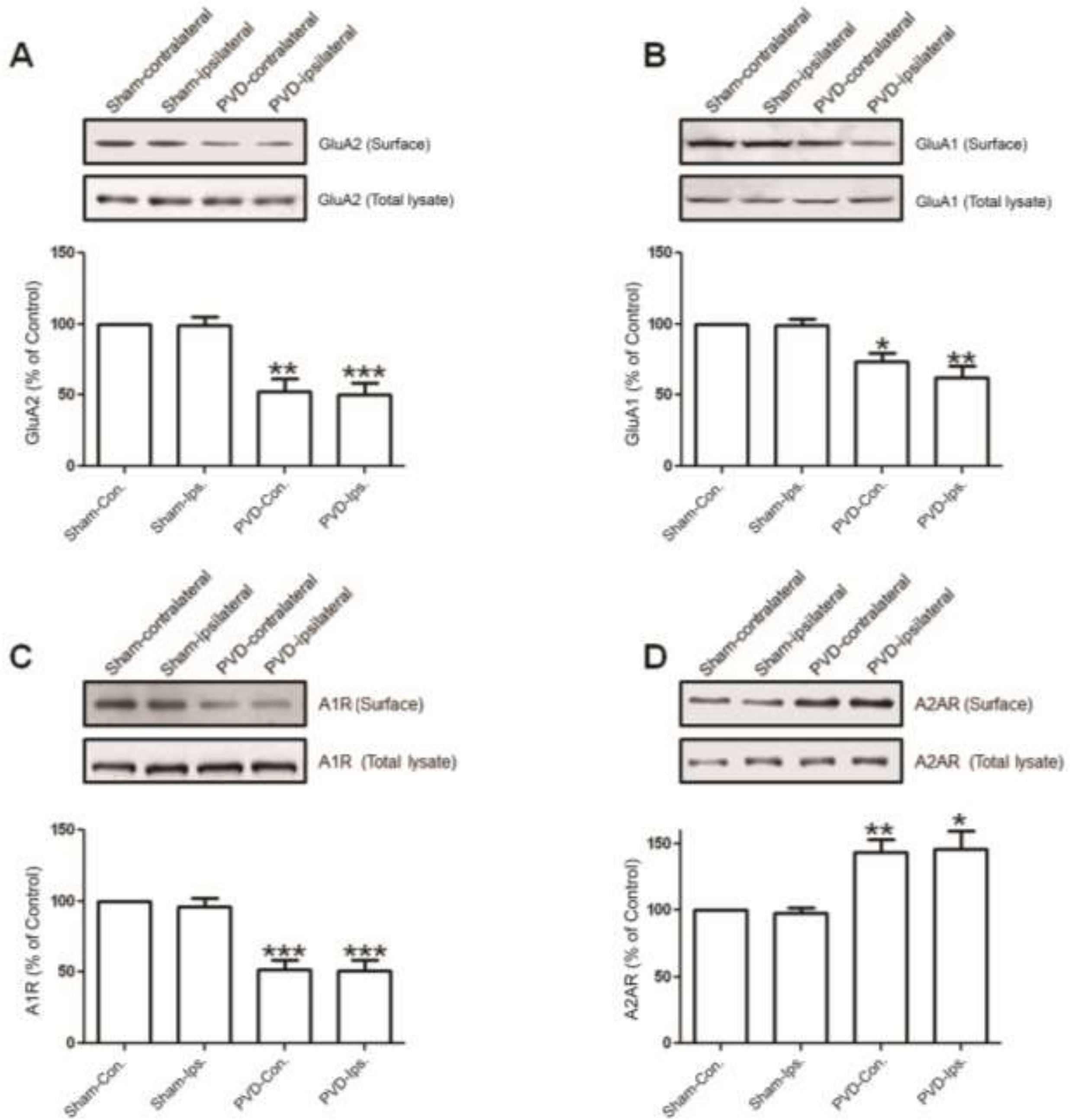


Figure 2.3.11 A focal cortical cerebral ischemia model with pial vessel disruption (PVD) injury affects expression of AMPARs and adenosine receptors in hippocampus. Two days after PVD or sham surgeries, hippocampal slices were prepared for biotinylation and subsequent immunoblotting, and some slices were used for electrophysiology (see Fig. 12). The resulting focal cortical lesions decreased surface expression of GluA2 (A), GluA1 (B), and A1R (C) but increased A2AR expression (D) in PVD at both ipsilateral and contralateral sides of the hippocampus compared to sham-operated animals. Values in summary bar charts represent mean±SEM (N=4 animals each), with $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

Figure 2.3.12

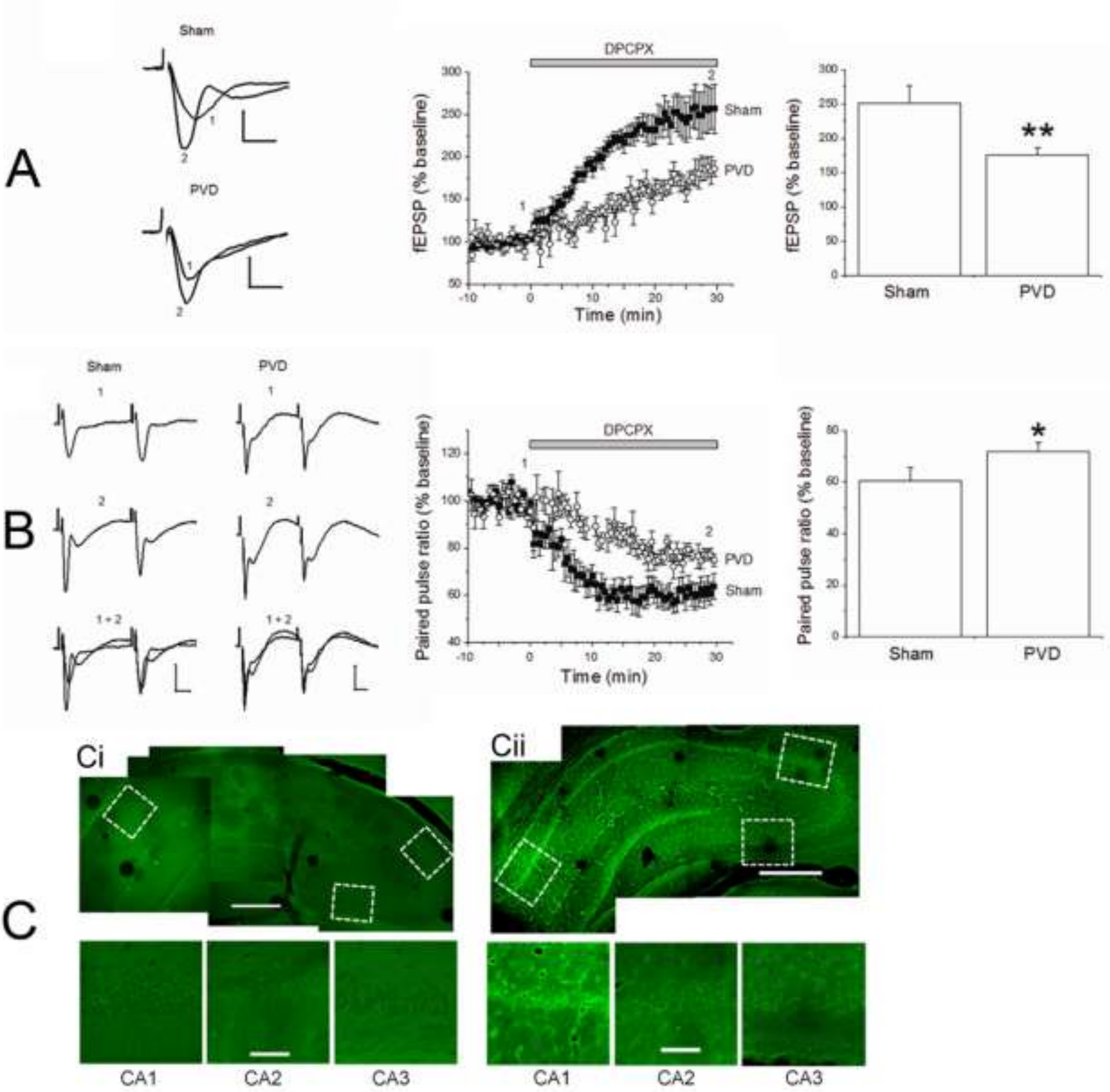


Figure 2.3.12 PVD model of focal cortical cerebral ischemia leads to increased synaptic depression in hippocampus. (A) Hippocampal slices from sham-operated or PVD-lesioned brains were exposed to 500nM DPCPX for 30min to assess the level of synaptic depression (reflecting adenosine tone). DPCPX induced greater synaptic transmission in sham vs. PVD hippocampal slices. (B) Responses to paired pulses (50ms apart) revealed greater paired-pulse depression in sham *vs.* PVD hippocampal slices. Values in A-B are means±SEM from 4 independent experiments (4 animals each), with $*P<0.05$ and $**P<0.01$ unpaired Student's *t*-test. Calibration: 0.5mV, 5ms in A, and 10ms in B. The numbers "1" and "2" associated with figure traces and time course charts correspond to fEPSPs at baseline and fEPSP in DPCPX, respectively. C. Confocal images of Fluoro-Jade B staining of Sham hippocampus (Ci) and PVD hippocampus (Cii). Images are from the ipsilateral side of sham surgery or PVD lesion, and are representative of slices from four different animal experiments. Scale bars in low and high magnification images are 500μm and 100μm. Boxed regions near CA1, CA2 and CA3 pyramidal body layers in low magnification

2.4. Discussion

In the hippocampus, adenosine has been implicated in neuroprotection through the A1Rs (Rudolphi, Schubert et al. 1992; Wardas 2002) and neuronal damage through the A2ARs (Rudolphi, Schubert et al. 1992; Cunha 2005) in neuronal insult conditions such as ischemia/hypoxia. However, the signal transduction pathways involved in these contrasting actions are not yet well understood. Acknowledging the contributions of glutamate and adenosine to synaptic depression in cerebral ischemia, we investigated the potential roles of AMPA glutamate receptors in A1R-mediated persistent synaptic depression, which we have termed *APSD*. Using biochemical, electrophysiological and confocal imaging techniques in combination with a Tat-peptide interference and shRNA genetic knockdown strategies, we discovered a novel functional and biochemical interaction between A1Rs and AMPARs that ultimately contribute to APSD in the hippocampus. Our results indicate that hippocampal APSD is mediated by clathrin-mediated, dynamin-dependent internalization of GluA2- and GluA1-containing AMPARs

after prolonged A1R stimulation. To further explore the molecular mechanisms of APSD induction and A1R-mediated AMPAR internalization, pharmacological inhibition or genetic knockdown of p38 MAPK and JNK was used. We show reduced p38 MAPK and/or JNK activity prevented GluA2, but not GluA1, AMPAR downregulation. This study suggests an important regulatory pathway with potential therapeutic targets to mitigate adenosine-induced hypoxic/ischemic damage to the brain.

The p38 MAPK and JNK have diverse biological functions, including regulation of gene expression, synaptic plasticity and cell survival (Cargnello and Roux 2011; Denise Martin, De Nicola et al. 2012), and are also implicated in internalization of epidermal growth factor receptor and AMPARs (Xiong, Kojic et al. 2006; Boudreau, Reimers et al. 2007; Lambert, Ameels et al. 2008; Lambert, Frankart et al. 2010). Since A1Rs and hypoxia lead to the activation of p38 MAPK and JNK in the brain (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007; Sanchez, Tripathy et al. 2012), we hypothesized that A1R-induced AMPAR endocytosis is dependent on p38 MAPK and JNK. Activation of p38 MAPK by the protein synthesis inhibitor anisomycin has been shown to induce clathrin-mediated internalization of GluA2 AMPARs (Xiong, Kojic et al. 2006), which is in accordance with our finding that A1R activation or hypoxic/ischemic insult leads to clathrin-mediated GluA2 internalization dependent on p38 MAPK and JNK activation. However, our results showed that pharmacological inhibition or shRNA knockdown of p38 MAPK and JNK prevented GluA2, but not GluA1 internalization, indicating that p38 MAPK and JNK could selectively target GluA2. In contrast, p38 MAPK, JNK, and extracellular signal-regulated kinase (ERK) phosphorylation was shown to be inversely proportional to the surface expression of GluA1 in the nucleus accumbens after cocaine challenge (Boudreau, Reimers et al. 2007).

Because AMPARs form functional hetero- or homo-oligomeric receptors, the subunit composition of each receptor is important in the regulation of the receptors (Optiz, Grooms et al. 2000). The C-terminus of each of the four AMPAR subunits are major regulatory sites for a variety of signaling proteins, including various phosphorylation sites for major serine/threonine kinases such as protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent kinase II (CaMKII) (Greengard, Jen et al. 1991;

Blackstone, Murphy et al. 1994; Tan, Wenthold et al. 1994; Wang, Dudek et al. 1994; Barria, Derkach et al. 1997; Barria, Muller et al. 1997; Mammen, Kameyama et al. 1997). AMPAR subunit phosphorylation, GluA1 in particular, potentiates channel activation by increasing single channel conductance, channel open frequency, and mean open time (Greengard, Jen et al. 1991; Blackstone, Murphy et al. 1994; Derkach, Barria et al. 1999; Banke, Bowie et al. 2000; Oh and Derkach 2005). Two major phosphorylation sites of GluA1, Ser831 and Ser845, targets of PKC/CAMKII or PKA, respectively, have been implicated in GluA1 surface expression and synaptic translocation, which are important in synaptic plasticity (Esteban, Shi et al. 2003; He, Lee et al. 2011). These phosphorylation sites are dynamically regulated by phosphorylation and dephosphorylation events, where dephosphorylation has been shown to involve protein phosphatase 1 (PP1) and calcineurin (PP2B), which promote GluA1 internalization and long term depression (Kameyama, Lee et al. 1998; Lee, Kameyama et al. 1998; Ehlers 2000; Lee, Barbarosie et al. 2000; Lee, Takamiya et al. 2003).

Since A1R stimulation leads to protein phosphatase 2A (PP2A) activation and translocation to membrane fractions (Brust, Cayabyab et al. 2006), we hypothesized that PP2A inhibition would prevent GluA1 internalization. We show that treatment of hippocampal slices with the PP2A inhibitors Okadaic Acid and Fostriecin prevented CPA-induced GluA1 and GluA2 internalization after CPA treatment. This is in accordance with other studies that show GluA1 phosphorylation states are integral in the regulation of GluA1 surface expression and function (Lee, Kameyama et al. 1998; Lee, Takamiya et al. 2003). We tested the hypothesis that CPA induces a reduction of phosphorylated Ser845 (pSer845) on the C-terminus of GluA1 and we show that high concentrations of CPA, the A1R agonist, induce a reduction in pSer845.

We initially sought to establish a functional and biochemical coupling between AMPARs and A1Rs in hippocampus, and found that GluA2 and GluA1 are physically associated with A1Rs but not with A2ARs. Since the C-terminus of AMPAR subunits contain binding sites for a complex array of signaling and binding proteins, it is possible that a direct or indirect protein-protein interaction exists between AMPARs and A1Rs. For example, the D2 dopamine receptors exhibit an indirect interaction with GluA2-containing

AMPARs, via direct interaction of the GluA2 C-terminus with N-ethylmaleimide-sensitive factor (NSF) to stabilize AMPAR surface expression (Henley, Nishimune et al. 1997; Nishimune, Isaac et al. 1998; Osten, Srivastava et al. 1998; Song, Kamboj et al. 1998; Zou, Li et al. 2005). In contrast to NSF, the scaffolding protein SAP-97 (Leonard, Davare et al. 1998; Sans, Racca et al. 2001) and the cytoskeletal proteins 4.1G/4.1N (Shen, Liang et al. 2000; Lin, Makino et al. 2009) directly interact with the GluA1 C-terminus to promote surface expression of GluA1-containing AMPARs. In addition, the endocytic adaptor protein 2 (AP2) (Lee, Liu et al. 2002), glutamate receptor interacting protein (GRIP) (Dong, O'Brien et al. 1997), and protein interacting with C kinase-1 (PICK1) (Staudinger, Zhou et al. 1995; Xia, Zhang et al. 1999) have all been shown to bind to the C-terminus of GluA2 and GluA3. However, it remains to be determined whether these interacting proteins are present in the same macromolecular protein complex as A1Rs and AMPARs to potentially regulate AMPAR internalization, synaptic depression and neurotoxicity following A1R stimulation by CPA or by excessive levels of adenosine during cerebral ischemia.

The cellular mechanisms underlying A1R-mediated changes in synaptic plasticity and neurotoxicity are still unclear. Our current findings indicate a dynamic physio-pathological adaptation of glutamatergic synapses to insults triggering a massive elevation of cerebral adenosine tone. We determined that prolonged A1R stimulation with CPA or in PVD focal cortical stroke model led to substantial APSD that did not recover following treatments with the A1R antagonist DPCPX. Although fEPSP recovery was observed in the presence of Tat-GluA2-3Y peptide, APSD was not abolished in Tat-GluA2-3Y-pretreated hippocampal slices. Nevertheless, these data indicate that GluA2-containing AMPARs are required for restoration of synaptic activity following prolonged exposure to adenosine during PVD cortical stroke. We found in earlier studies (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007) that CPA applied for shorter duration (10 min) and at 10-fold lower concentration (40-50nM) produced lower levels of APSDs (~25% compared to 50-60% APSDs in present study using 500nM CPA for 30min). We also observed that 50nM CPA did not significantly alter levels of GluA1 phosphorylation at Ser845 (Figure 2.3.7D), indicating that more prolonged A1R stimulation may be required for robust changes in surface AMPARs, such as during cerebral ischemic insults that trigger a massive surge of extracellular adenosine. This prolonged A1R stimulation led to clathrin-mediated

internalization of GluA2-containing AMPARs. The resultant decrease in both GluA2-containing AMPARs and A1Rs as well as the more pronounced APSDs, may contribute to the increased susceptibility of hippocampal CA1 pyramidal neurons to ischemic damage following PVD. Interestingly, we found that p38MAPK and JNK inhibitors selectively reduced GluA2 AMPAR internalization, while PP2A inhibitors caused similar reductions in clathrin-mediated endocytosis of GluA2 and GluA1 AMPARs. Our results also showed that the neuroprotective and inhibitory adenosine A1 receptor surface expression decreased, while the surface levels of the neurotoxic and excitatory adenosine A2A receptors increased during PVD. These results indicate that APSD could mediate neuronal damage in subsequent periods following PVD cortical stroke, although it appears to afford neuronal protection during acute neuronal insult, such as acute hypoxia. However, future studies are required to establish the role of APSD in adenosine-induced neurodegeneration.

It is also possible that chronic and acute A1R activation could exert a different influence on synaptic plasticity. For example, chronic exposure to CPA enhanced learning in mice (Von Lubitz, Paul et al. 1993), whereas acute administration of CPA impaired learning capacity (Normile and Barraco 1991). In middle-aged rats (7-10 months old), long term potentiation (LTP) was impaired (Rex, Kramar et al. 2005), which was consistent with higher adenosine tone that likely enhanced disinhibition of synaptic transmission in aged rats (Sebastiao, Cunha et al. 2000). In line with these behavioral findings, acute administration of an A1R antagonist enhanced, while endogenous adenosine reduced LTP, LTD and depotentiation (de Mendonca and Ribeiro 1994; de Mendonca, Almeida et al. 1997; de Mendonca and Ribeiro 2000). LTP was also inhibited by the adenosine uptake blocker nitrobenzylthioinosine, presumably by increasing extracellular adenosine (de Mendonca and Ribeiro 1994). Cunha and colleagues have also reported previously that the A2ARs play a prominent role over A1Rs in regulating hippocampal LTP triggered by electrical stimulation in adult and aged rodents (Costenla, Cunha et al. 2010; Costenla, Diogenes et al. 2011). This requires an A2AR-regulated and NMDAR-mediated enhancement of LTP (Rebola, Lujan et al. 2008). In addition, Dias and colleagues (Dias, Rombo et al. 2013) recently reported that oxygen glucose deprivation unmasks a novel form of long term potentiation mediated by increased expression of

GluA2-lacking AMPARs and requiring the stimulation of A2ARs, which is opposite to the role of A1Rs in APSD during PVD and hypoxia (present study). Our findings obtained from juvenile rats indicate a novel form of long lasting synaptic depression, known as APSD, which is triggered by prolonged A1R stimulation, involving activation of p38 MAPK, JNK and PP2A, and clathrin-mediated endocytosis of GluA2-containing AMPARs. We propose that p38 MAPK and JNK inhibitors would be potent inhibitors of the A1R-mediated excitotoxic potential, by decreasing GluA2 internalization and promoting the neuroprotective GluA2-containing AMPARs on neuronal membranes. Therefore, both A2ARs and A1Rs contribute to adenosine neuromodulation of glutamatergic synapses by increasing GluA2-deficient AMPARs and inducing LTP (for A2ARs, see (Dias, Rombo et al. 2013)) and increasing GluA2 and GluA1 endocytosis to produce APSD (for A1Rs, present study). Since previous reports suggested that A2ARs are increased while A1Rs are decreased in middle aged rats (Cunha, Constantino et al. 1995; Sebastiao, Cunha et al. 2000; Rebola, Sebastiao et al. 2003), it remains to be established whether this novel form of long lasting synaptic depression is maintained in middle aged animals.

Our present study supports the hypothesis that prolonged A1R activation during hypoxia or focal cortical ischemia causes clathrin-mediated GluA2 and GluA1 AMPAR endocytosis and persistent synaptic depression, which could contribute significantly to increased neuronal death. The novel signaling complex formed by A1Rs, GluA2-containing AMPARs, and MAPKs (p38 MAPK and JNK) represents an important mechanism of ischemic damage that may provide effective therapeutic targets in cerebral ischemia.

CHAPTER 3

Casein Kinase 2 Regulates Equilibrative Nucleoside Transporter and Adenosine A1 Receptor in Rat Hippocampus

Zhicheng Chen, Jocelyn Stockwell, Nicole L. Longmuir, and Francisco S. Cayabyab

Jocelyn Stockwell contributed to the acquisition of confocal images of propidium iodide staining. Nicole L. Longmuir contributed some initial biochemical studies including membrane fractionation of ENT1. All other data were collected by Zhicheng Chen.

Abstract

Equilibrative nucleoside transporters (ENTs) and adenosine A1 receptors (A1Rs) are widely expressed in the hippocampus, acting to regulate extracellular adenosine levels and induce synaptic depression, respectively, during cerebral ischemia. However, the cellular mechanisms that control the cell surface expression of ENTs and A1Rs in the brain remain poorly resolved. Since ENTs contain consensus sites for casein kinase 2 (CK2) phosphorylation, we hypothesized that ENT and A1R interactions and CK2 inhibition are involved in A1R-dependent downregulation of ENT surface expression during hypoxia. Coimmunoprecipitation from Sprague-Dawley rat hippocampal brain homogenates and confocal imaging microscopy of primary cultured hippocampal neurons revealed physical associations of ENTs with A1Rs, but not with A2A receptors. Using whole lysates and membrane fractions from hippocampal brain slices and a phospho-specific antibody to immunoprecipitate the phospho-Serine254-ENT1 (pSer254-ENT1, a known CK2 target), we determined that ENT1 was constitutively phosphorylated. Several CK2 inhibitors (TBB, DMAT, and DRB) but not the ENT1-selective inhibitor NBTI reduced pSer254-ENT1 levels in whole lysates. DRB also decreased, while CK2 activator spermine increased, the surface expression of pSer254-ENT1 in biotinylation assays of hippocampal brain slices. Moreover, biotinylation of cultured hippocampal neurons revealed that ENT1 and ENT2 surface expression was downregulated by CK2 and ENT inhibitors and by the A1R agonist CPA, but not in the presence of the A1R antagonist DPCPX. Pretreatments of hippocampal slices with CK2 or ENT inhibitors also enhanced hypoxia-mediated ENT and A1R surface expression downregulation. CK2 inhibitors reduced neurodegeneration caused by hypoxic insult in hippocampal slices, suggesting CK2 inhibitors are neuroprotective. These results indicate that CK2-induced and A1R-linked ENT trafficking represents an important regulatory mechanism of hypoxic/ischemic hippocampal brain damage.

3.1 Introduction

During cerebral ischemia or stroke, the level of extracellular adenosine is elevated, with two possible sources: adenosine release from ischemic brain cells and derived from extracellular ATP metabolism (Dunwiddie and Masino 2001; Benarroch 2008). Extracellular adenosine acts as a neuromodulator by inhibiting excitatory synaptic transmission through the activation of adenosine A1 receptors (A1Rs), and may also act as an endogenous neuroprotectant by reducing glutamate excitotoxicity (Dunwiddie and Masino 2001). It has been reported that selective A1R stimulations or brief hypoxia induces profound synaptic depression in the hippocampus that is partially mediated by p38 MAPK and JNK activation (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). The inhibitory effect of A1R stimulation on synaptic transmission was mediated primarily by a presynaptic mechanism, since A1R agonists increased paired-pulse facilitation. However, after prolonged washout of the A1R agonist, a persistent synaptic depression remained even after paired pulse facilitation has recovered back to baseline (Brust, Cayabyab et al. 2007). This indicated that the actions of adenosine are not all presynaptic, but a postsynaptic action is also included. Consistent with this suggestion, the A1Rs have been found to be distributed on the surface of somatodendritic structures in the hippocampus as shown by immunofluorescence and electron microscopy studies (Ochiishi, Chen et al. 1999), and biochemical data also demonstrated a highly localized distribution of A1Rs in the active zone and postsynaptic density in hippocampal synapses (Rebola, Pinheiro et al. 2003). However the regulatory mechanisms controlling adenosine tone and the postsynaptic actions of A1R stimulation in an ischemic environment require further investigation.

Facilitated diffusion of adenosine across cellular membranes via nucleoside transporters is a mechanism by which intracellular and extracellular concentrations of adenosine are controlled (Dunwiddie and Masino 2001). Two families of nucleoside transporters have been identified in mammalian cells: Na^+-dependent concentrative nucleoside transporters (CNTs) and Na^+-independent equilibrative nucleoside transporters (ENTs) (Baldwin, Beal et al. 2004; Kong, Engel et al. 2004). Six subtypes of CNTs (CNT1-6) and four subtypes of ENTs (ENT1-4) have been further identified. The present study focuses on the two best-characterized equilibrative nucleoside transporters,

ENT1 and ENT2, which are ubiquitously expressed throughout the body. These isoforms are highly expressed in pyramidal neurons of the hippocampus, an area of the brain that is highly susceptible to ischemic damage (Anderson, Xiong et al. 1999).

Nucleoside transporters mediate adenosine transport across cell membranes and are therefore major regulators of intracellular and extracellular adenosine levels. However, little is known about how ENTs are regulated in the hippocampus. It has been shown that nucleoside transporters are subject to regulations by intracellular signaling pathways, and both activators and inhibitors of protein kinase C (PKC), protein kinase A (PKA) and CK2 have been shown to alter the transport function and membrane expression of ENTs in a variety of cell types (Coe, Zhang et al. 2002; Stolk, Cooper et al. 2005), but whether these signaling molecules regulate ENT function in the brain remains unclear. Both ENT1 and ENT2 have 11 transmembrane domains and a large intracellular loop between transmembrane domains 6 and 7, which contains protein kinase CK2 phosphorylation consensus sites, suggesting that these transporters are potentially regulated by CK2. Rat ENT1 contains CK2 phosphorylation consensus sites at Thr-248 and Ser-254, while rat ENT2 contains one CK2 phosphorylation site at Thr-236 (Kiss, Farah et al. 2000; Handa, Choi et al. 2001). In addition, two splice variants of mouse ENT1 have been identified that differ in their amino acid sequence such that the shorter variant lacks one of the accepted CK2 phosphorylation consensus sites (Ser-254). Hence, the shorter variant has a reduced transport activity relative to the other variant with both consensus sites (Kiss, Farah et al. 2000; Handa, Choi et al. 2001). However, it is not yet established whether the activities of CK2 and/or A1Rs can regulate the subcellular localization of ENTs in the hippocampus.

The present study was undertaken to determine whether phosphorylation of ENTs by CK2 is a major determinant of ENT surface expression in rat hippocampal neurons and brain slices. The results show that activations of CK2 increased while inhibition of CK2 decreased the phosphorylation and surface expression of ENTs in cultured hippocampal neurons and hippocampal brain slices, and levels of co-precipitated A1Rs and ENTs were reduced in the presence of CK2 inhibitors. In addition, direct inhibitions of ENTs or direct A1R stimulations led to an A1R-mediated reduction in ENT surface

expression, which was consistent with the direct coupling of ENTs and A1Rs. Finally, hypoxia caused significant downregulation of ENT and A1R surface expression, and this downregulation effect was further enhanced by preincubation of hippocampal brain slices with CK2 or ENT blockers. CK2 inhibitors can rescue hypoxia-induced neurodegeneration, suggesting CK2 inhibitors are neuroprotective in the rat hippocampus. Together, these results indicate that functional and biochemical interactions between ENTs and A1Rs and their surface densities depend on CK2 activity, which represents an important mechanism for targeting CK2 activity and A1R-ENT signaling complex in hypoxic/ischemic brain damage.

3.2 Materials and Methods

Ethics statement

All animals were treated according to guidelines of the Canadian Council for Animal Care (CCAC) under the supervision of the University of Saskatchewan Committee on Animal Care and Supply (UCACS) under animal protocol approval number 20070090.

3.2.1 Hippocampal slice preparations and treatments

Young male Sprague-Dawley rats at postnatal day 18-30 (P18-30) (Charles River, Canada) were anesthetized with halothane and decapitated according to protocols approved by the UCACS. Brains were quickly extracted and placed in ice-cold oxygenated dissection medium containing the following (in mM): 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 1h at room temperature (RT) in artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.0 $CaCl_2$, and aerated with 95% O_2/5% CO_2. Hippocampal slices were then incubated in ACSF solution at RT (22-24°C) with the various drug treatments: dipyridamole, DPY (10μM); S-(4-Nitrobenzyl)-6-thioinosine, NBTI (100 nM); dimethylaminotetrabromobenzimidazole, DMAT (5 μM); tetrabromobenzotriazole, TBB

(10 μM); spermine tetrahydrochloride (300 μM, Tocris) or dichlororibofuranosylbenzimidazole, DRB (100 μM) for 1.5h. Brain slices were incubated for another 20min in normoxic ACSF or hypoxic ACSF (i.e., ACSF bubbled for 15min with 95% N_2/5% CO_2) as previously described (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). DMSO was used as a vehicle control. All chemicals were obtained from Sigma-Aldrich (except when mentioned otherwise) and diluted in DMSO before being added to ACSF. The final concentration of DMSO was always <0.1% of the final solution.

3.2.2 Hippocampal lysate preparation, biotinylation assay, and western blotting

Following the treatments described above, hippocampal slices were lysed and separated into membrane and cytosolic fractions, which were later analyzed by quantitative western blotting technique as previously described (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). In brief, the hippocampal tissue slices were lysed in a solubilization buffer (30min) that contained 50mM Tris (pH8.0), 150mM NaCl, 1mM EDTA, 1mM NaF; protease inhibitors: 1mM PMSF, 10μg/μL aprotinin, 10μg/mL pepstatin A, 10μg/mL leupeptin, 2mM Na_3VO_4, 20mM sodium pyrophosphate, 3mM benzamidine hydrochloride, 4mM glycerol 2-phosphate. The tissue homogenates were then centrifuged at 1,000xg (5min at 4°C) to remove cellular debris. Membrane and cytosolic fractions were separated by centrifugation at 13,000xg for 1h 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). Whole lysates from hippocampal slices were prepared using solubilization buffer containing 1% NP-40 and protease inhibitors (Cayabyab, Gowribai et al. 2013). Protein concentrations were determined using Bradford assays with the Bio-Rad dye.

For biotinylation experiments, hippocampal slices or 7 day old cultured hippocampal neurons (see below) were incubated with 1.2μM tetrodotoxin in ACSF whenever DPCPX was used to prevent DPCPX-mediated glutamate release, which could confound the direct effects of drug inhibitors on ENT and A1R surface localization. After treatments, hippocampal slices or neurons were then cooled to 4°C (20-30min), and then washed

with ice-cold ACSF before biotinylation. Slices or neurons were then incubated with 1 mg/ml NHS-SS-Biotin (Pierce) at 4°C for 45 min. Rinsing the tissue with quenching buffer stopped the reaction. Slices were then transferred into lysis buffer containing protease inhibitors and 1% NP-40 detergent (as above). After determining the protein concentrations using the Bradford Assay with the DC Protein assay dye (Bio-Rad), equal amounts of protein lysates (200-500μg) were diluted in lysis buffer, and biotinylated proteins were incubated overnight with streptavidin beads. The beads were then washed 4-6 times the next day with lysis buffer containing 0.1% NP-40. The proteins were eluted by adding 50μl of 1X Laemmli sample buffer and boiling the samples at 95°C for 10min. Similarly, hippocampal lysates from brain slice homogenates and membrane and cytosolic fractions were diluted with 1X Laemmli sample buffer and boiled for 10min. The samples were run on 10% SDS-PAGE gels, and the resulting blots were probed with the appropriate primary and secondary antibodies. The primary antibody dilutions are as follows: polyclonal rabbit anti-ENT1 (1:1000; Millipore), polyclonal rabbit anti-ENT2 (1:1000; Abcam), rabbit anti-phospho-Serine 254-ENT1 (1:1000, Abgent), mouse anti-phospho-Serine antibody (1:1000, Santa Cruz Biotechnology), polyclonal rabbit anti-A1R (1:1000; Sigma), polyclonal rabbit anti-A2AR (1:1000, Sigma), monoclonal mouse anti-GAPDH (1:2000; Millipore). Following four washes (15min each) with TBST, the membranes were incubated with rabbit, mouse or goat horseradish peroxidase-conjugated secondary antibody against IgG (1:000; Santa Cruz, 1:3000 for GAPDH) in 5% non-fat milk (1h, room temperature). The membranes were then washed 4 times (15min) with TBST, and proteins were visualized using enhanced chemiluminescent reagent (Santa Cruz). Surface labeled proteins (i.e., biotinylated) were normalized to their respective proteins present in whole lysates, since the levels of ENT1, ENT2 and A1R in hippocampal whole lysates remained constant independent of the type of treatments. For membrane and cytosolic fractions, protein signals were quantified by normalizing all bands with the GAPDH protein band signals.

3.2.3 Immunoprecipitation

To examine interactions of ENT1 and ENT2 with A1R or A2AR, immunoprecipitation was performed by first incubating 500μg extract from hippocampal homogenates with the

antibody corresponding to the protein being immunoprecipitated (overnight; 4°C). The antibodies used were: 2-4μg rabbit anti-ENT1 (Millipore), 2-4μg rabbit anti-ENT2 (Abcam), 4μg rabbit anti-A1R (Sigma), and 4μg rabbit anti-A2AR (Sigma). Negative controls included omitting the immunoprecipitating antibodies and replacing with 0.2μg rabbit IgG (Sigma). After overnight incubation with primary antibodies, then either anti-mouse IgG (whole molecule) agarose (Sigma), anti-rabbit IgG (whole molecule) agarose (Sigma), or protein A/G agarose (Santa Cruz) beads were added to the homogenates for either an additional 3h (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 65μl of 1x Laemmli sample buffer (Bio-Rad), boiled for 10min, and resolved in polyacrylamide gels. Proteins were then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and proteins were visualized using enhanced chemiluminescence.

To determine whether CK2 phosphorylates ENT1 proteins at serine residues, we first performed immunoprecipitation of phospho-serine proteins using 2μg of mouse monoclonal IgM anti-phospho-serine antibody (Millipore) with 300μg lysates from hippocampal membrane fractions prepared from control slices or slices pre-treated with the CK2 inhibitor TBB (20μM for 1.5h). The resulting blots were probed with anti-ENT1 antibody (Millipore), which showed that ENT1 proteins were reduced in phosphor-Serine by immunoprecipitation (see Figure3.3.3). Conversely, ENT1 was also immunoprecipitated using 2μg of rabbit polyclonal ENT1 antibody (Millipore) from hippocampal membrane lysates, and the presence of phospho-serines in these ENT1 immunoprecipitates was also showed with the anti-phospho-serine antibody (data not shown). We then confirmed using hippocampal whole cell lysates whether the CK2 inhibitor TBB reduced ENT1 phosphorylation at the ser-254-ENT1 site, a known CK2 target (Kiss, Farah et al. 2000; Handa, Choi et al. 2001; Bone, Robillard et al. 2007). We immunoprecipitated phospho-Ser254-ENT1 using 2μg of the rabbit polyclonal anti-phospho-Ser254-ENT1 antibody (Abgent), and probed the resulting blots with anti-ENT1 (Millipore). Reverse immunoprecipitation was also performed to confirm that the ENT1

immunoprecipitates (2μg of anti-ENT1 antibody from Millipore) contained the phospho-Ser254-ENT1 (Abgent) in immunoblots. Normal rabbit IgG or mouse IgG (0.4μg, Santa Cruz Biotechnology) was used as a negative control for the immunoprecipitating antibodies. After overnight incubation, the immune complexes were captured with protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 4h at 4ºC. Agarose beads were then collected by pulse spins, and washed four times with wash buffer (solubilization buffer containing 0.1% NP-40). Immunoprecipitated proteins from the agarose beads were eluted and detected by western blotting as described above.

3.2.4 Hippocampal neuron culture, immunocytochemistry, and image acquisition and analysis

Rat hippocampal neurons were cultured as described by others (Kaech and Banker, 2006) and immunocytochemistry was performed 10-14 days after plating. In brief, low-density hippocampal neurons ($5x10^4$ cells/35 mm culture dishes) from 17-18 day old embryonic rat brains are grown on polylysine-coated coverslips, which are suspended above a one week old astrocyte feeder layer. The hippocampal neuron/astrocyte cultures were kept in Neurobasal medium (Invitrogen) supplemented with 2% B27 serum-free supplement (Invitrogen), 1% GlutaMAX-I supplement (Invitrogen), containing 1% penicillin-streptomycin (100 U/mL) (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Every 2–3 days half of the medium was replaced with fresh medium. For immunocytochemistry, the hippocampal neurons were treated with pharmacologic agents tetrodotoxin (TTX, 0.5μM), bicuculline (50μM), strychnine (1μM) and D-APV (50μM) for 20-30min, to block neural activity, $GABA_A$, glycine, and N-methyl-D-aspartate (NMDA) receptors, respectively.

To assess the possible colocalization of A1Rs and ENTs, hippocampal neurons were washed three times with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde, washed three times with PBS, permeabilized for10 min with 0.25% Triton X-100 in PBS, and then blocked for 1h at RT with PBS containing 5% bovine serum albumin (BSA, Sigma). The neurons were triple labeled by overnight incubation (at 4°C) with the following antibodies and dilutions: rabbit anti-ENT1 (1:200, Abcam) or rabbit anti-

ENT2 (1:200, Abcam); goat anti-A1R (1:200, Santa Cruz) or mouse anti-A2AR (1:200, Santa Cruz); and chicken anti-MAP2 (1:2000, Abcam). Antibodies were diluted at 1:200 in blocking buffer followed by brief washes (three times; 10min each) and incubation for 1h at room temperature with the appropriate secondary antibodies, including Alexa Fluor 555-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated donkey anti-mouse or anti-goat secondary antibodies (Invitrogen), and Alexa Fluor 633-conjugated anti-chicken secondary antibody (all at 1:1000). All neuronal nuclei were labeled with Hoechst (Sigma). Finally, the coverslips were mounted on newly cleaned slides using Prolong Gold Antifade Reagent (Invitrogen).

As previously described (Cayabyab, Gowribai et al. 2013), confocal images were acquired with the Zeiss Zen 2009 software (version 5.5 SPI) of the Zeiss LSM 700 laser scanning confocal microscope (Carl Zeiss) equipped with a 405nm, 488nm, 555nm and 639nm solid state laser lines. For all the quantitative colocalization analyses, we used a Zeiss Plan-Apochromat 63X/1.6 oil objective lens and identical acquisition parameters (laser intensities, detector sensitivity, digital offset, and microscope pinhole set to 1 Airy unit) for a given set of labeled neurons without over- or under-saturation of the acquired signals. Each fluorophore was imaged individually using the multi-track channels to prevent dye bleed. Images were exported as 8 bit TIFF files, and the degree of colocalization between fluorescent probes was quantified using the Intensity Correlation Analysis plug-in in ImageJ (software download from NIH, version 1.44f), which calculated both the Pearson's correlation coefficients and Mander's coefficients. We report the Pearson's coefficients in this study since the ratio of fluorescent signals in the two channels were different from unity. Negative Pearson's coefficients indicate the two signals do not colocalize, while values closer to 1 indicate strong colocalization between the two particles. Colocalization analysis was obtained from three 3 different hippocampal neuronal cultures.

3.3.5 Propidium iodide staining

The methods used to stain hippocampal slices with propidium iodide (PI) were adopted from a paper by Pugliese et al. (2009). Following equilibration of hippocampal

slices for 1h after slicing, the following drug treatments were added: the CK2 inhibitors DMAT and TBB, and the CK2 activator spermine. Slices that were treated with spermine were also treated with D-APV to prevent aberrant NMDAR activation prior to spermine for 1h. Hippocampal slices were then subjected to a 20min hypoxic insult by replacing oxygenated ACSF with hypoxic ACSF that was bubbled with 95% N_2 / 5% CO_2 prior to and continuously throughout the hypoxic insult. After 20min hypoxia, ACSF was replaced with normoxic ACSF and the slices were incubated at room temperature for 3h. During the final 1h incubation period, 5 µg/ml propidium iodide (Sigma-Aldrich) was added to the ACSF. Following the incubation period, slices were rinsed thoroughly in ACSF and then fixed in 4% paraformaldehyde at 4°C overnight. In the following day, slices were washed 3 x 15min in 1X PBS and then mounted on glass microscope slides (VWR) and sealed using Prolong Gold Antifade Reagent (Invitrogen). Upon the addition of PI, all subsequent procedures were done in the dark to prevent photobleaching.

Hippocampal slices were imaged using a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss, Germany) using green light (543nm) to induce PI fluorescence. The entire hippocampus was imaged in pieces using a 10x objective lens, and images of CA1 pyramidal neurons were captured using the Zeiss Plan-Apochromat 63x/1.6 oil objective lens (Carl Zeiss). CA1 images were acquired as Z-stack images of 200µm depth into the hippocampal slice to the outer top of the slice, with each Z-stack image taken at 2µm (total 100µm). Two Z-stack images were taken along the CA1 region in each slice.

Data were collected using Zeiss Zen 2009 v. 5.5 software (Carl Zeiss) and analyzed using ImageJ (NIH, public domain). Z-stack images closest to the outer top and bottom of the hippocampal slices were not analyzed, as the neuronal damage in those areas was artificially enhanced by the slicing procedure. The inner-most 20µm segments (~100µm down) were combined as maximum intensity projections and intensities were compared between treatment groups. Densitometry analysis was performed on CA1 Z-stack maximum intensity projection images, and densitometry values were normalized to time control slices that were treated along with each experiment. Data was graphed as a percentage of the time control value and analyzed for significance against this control

value (100%). Full hippocampal images were assembled as montages of the entire hippocampal slice using Adobe Photoshop CS6 (Adobe Systems, Mountain View, CA).

3.2.6 Statistical analysis

All statistical tests were performed with GraphPad Instat3 version 3.00 for Windows 97 (GraphPad Software, San Diego, CA). Protein densitometry values and Pearson correlation coefficients were compared and statistical significance ($P < 0.05$) was assessed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test. Student's paired t-test was also used, as appropriate, with $P<0.05$ considered significant.

3.3 Results

3.3.1 ENTs functionally interact with A1Rs, but not with A2ARs

Since ENTs, A1Rs and A2ARs are expressed in the hippocampus, we first tried to determine whether these proteins are localized in the same signaling protein complex to be able to respond rapidly to changes in extracellular adenosine tone, such as an elevation during hypoxic/ischemic insults. Immunoprecipitation with ENT1 or ENT2 antibodies using brain homogenates from hippocampal brain slices and subsequent immunoblotting with rabbit polyclonal anti-A1R or anti-A2AR antibodies revealed the presence of A1Rs in both ENT1 and ENT2 immunoprecipitates (Figure 3.3.1A, top two panels), while A2ARs were absent. These A1R protein bands were not present when the immunoprecipitating antibody was omitted (Figure 3.3.1A, lane 1). In addition, we found that A1R (but not A2AR) immunoprecipitates contained ENT1 and ENT2 (both ~50 and ~60 kDa bands) in reverse coimmunoprecipitation from hippocampal brain lysates (Figure 3.3.1A, bottom two panels). These data suggested that ENTs and A1Rs physically interact with each other in the rat hippocampus, but future studies are required to further characterize the nature of interactions between ENTs and A1Rs. Since hypoxia or ischemia are known to elevate extracellular adenosine levels and consequently stimulate adenosine receptors (Fowler 1993; Fowler, Gervitz et al. 2003; Gervitz, Davies et al. 2003), and having found a novel interaction between A1Rs and ENTs, we next studied whether A1R stimulation affects the surface localization of ENTs in primary cultures of hippocampal neurons. After prolonged incubations of hippocampal neurons with the selective A1R agonist (CPA, 500nM for 45min), subsequent biotinylation assays were performed to label cell surface proteins. As shown in Figure 3.3.1B, CPA decreased the biotinylated (surface) levels of ENT1 compared to DMSO control, and the A1R antagonist DPCPX prevented the CPA-induced downregulation of ENT1 surface expression (summarized in Figure 3.3.1B, bottom). In contrast, the ENT1 levels in whole hippocampal cell lysates were not significantly altered (Figure 3.3.1B, bottom blots). These data indicated that A1Rs are not only functionally coupled, but also biochemically linked with ENTs in hippocampal neurons.

Figure 3.3.1

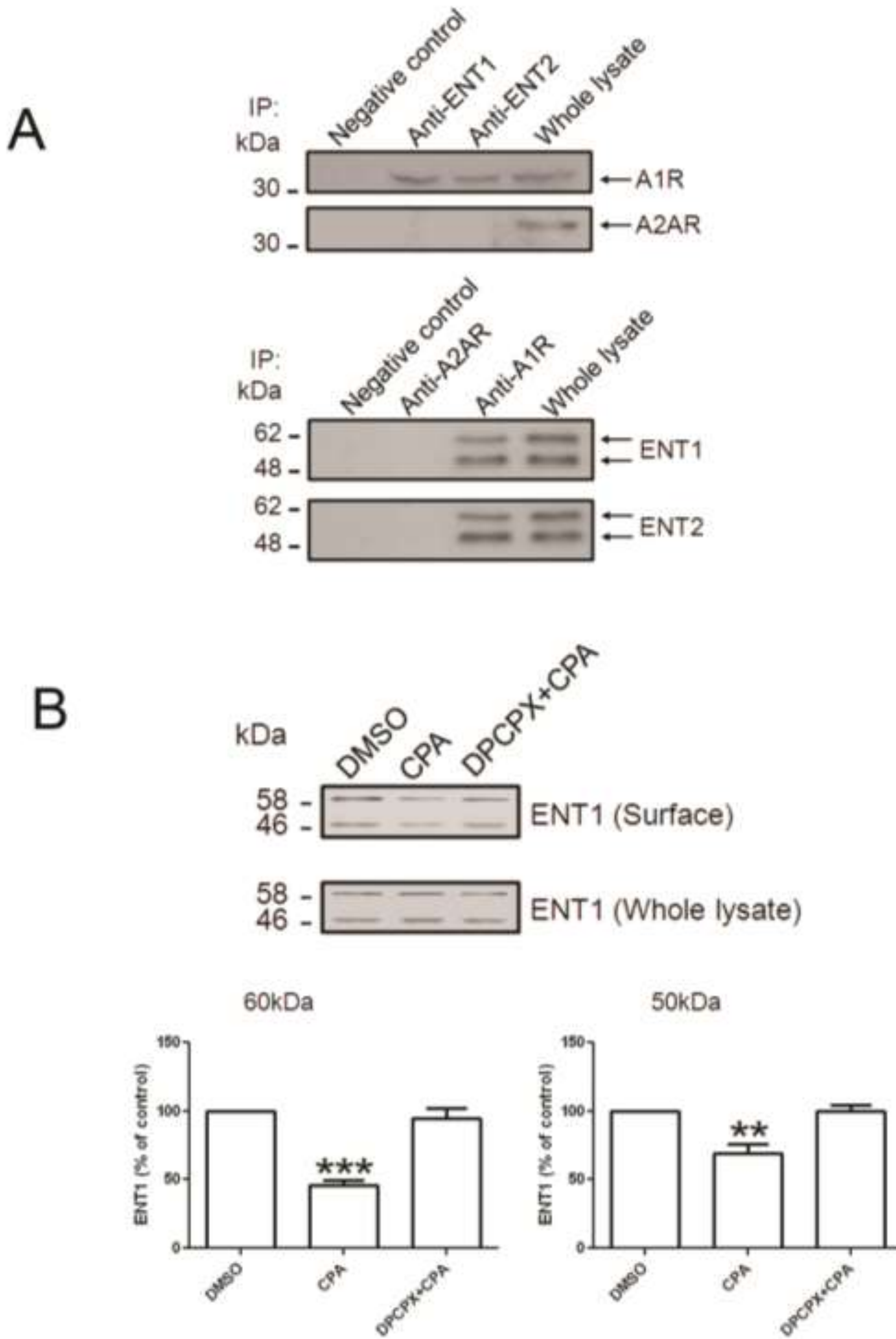


Figure 3.3.1 ENT1 and ENT2 selectively form a physical complex with A1Rs in hippocampal brain slices and A1R activation or inhibition affects ENT1 surface expression in cultured hippocampal neurons. (A) Using hippocampal whole lysates, immunoprecipitation with a rabbit polyclonal ENT1 antibody (lane 2) or rabbit polyclonal ENT2 antibody (lane 3) revealed co-precipitation with A1Rs (first blot) but not with A2ARs (second blot). Negative control (lane 1) indicates omission of the immunoprecipitating antibodies, and rabbit IgGs were used instead. The A1R protein band corresponds to ~37 kDa, and the A2AR band signal is near the predicted size of 45 kDa. Immunoprecipitation with a rabbit polyclonal A2AR antibody (lane 2) or with rabbit polyclonal A1R antibody (lane 3) revealed co-precipitation of ENT1 (third blot) and ENT2 (fourth blot). ENT1 and ENT2 double bands were detected near 50 kDa and 60 kDa. Above immunoprecipitation studies are from three independent experiments, using hippocampal brain lysates from P18-28 day-old male rats. (B) Primary hippocampal neurons were incubated with neural activity inhibitors (see Materials and Methods) before applying DPCPX (500nM) and CPA (500nM) treatments. After these treatments (45min), hippocampal neurons were subjected to biotinylation followed by western blotting to assess the surface expression of ENT1. Surface (biotinylated) ENT1 was detected with rabbit anti-ENT1 (Millipore), and surface protein signals were normalized to whole cell lysate signals. Values in bar charts are means±SEM, n=3, **P<0.01, ***P<0.001 vs. DMSO control.

3.3.2 ENTs colocalize with A1Rs, rather than with A2ARs

In addition to coimmunoprecipitation, we performed immunocytochemistry and confocal imaging analyses to determine the subcellular localization of the potential interactions between A1Rs and ENTs in hippocampal neurons. As shown in Figure 3.3.2A-B, A1Rs were found in somatodendritic compartments, which confirm previous results from immunofluorescence, electron microscopy, and biochemistry techniques (Ochiishi, Chen et al. 1999; Rebola, Pinheiro et al. 2003). Our results show that A1Rs colocalize in the somas and dendrites with both ENT1 and ENT2 (Figure 3.3.2A-B). In contrast, the A2ARs did not colocalize with either ENT1 or ENT2 (Figure 3.3.2C-D), which is consistent with our previous results indicating a lack of coimmunoprecipitation between A2ARs and ENTs. Determination of the Pearson correlation coefficients also confirmed that A1Rs, but not A2ARs, showed similar quantitative levels of interaction with ENT1 and ENT2, as summarized in Figure 3.3.2E. Thus, ENT1 and ENT2 appear to interact selectively with A1Rs. Future studies will be needed to determine whether these interactions are direct or indirect, and to determine the major binding domains for this interaction.

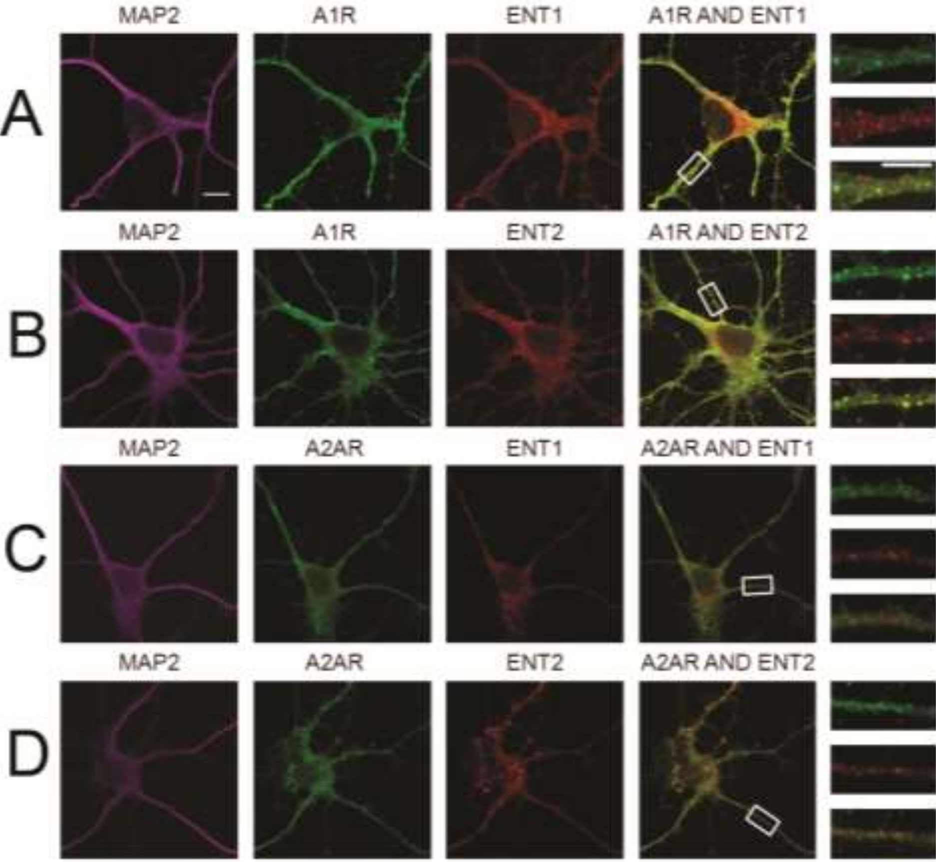
3.3.3 Inhibitors of CK2 decreased, activator of CK2 increased, phosphoSer254 ENT1 in rat hippocampal brain slices

ENT1 and ENT2 contain cytoplasmic serine and threonine residues that are potential candidates for CK2 phosphorylation with the consensus sequences S/TXXE/D, with serine preferred over threonine (see Table 3.1). Using the anti-pSer254 ENT1 antibody in our immunoblot analyses, we then sought to determine the effects of various classes of CK2 inhibitors on ENT1 expression in membrane fractions. As shown in Figure 3.3.3A (top blot), the 50 kDa and 60 kDa ENT1 bands were shown to be constitutively phosphorylated at Ser254 in control (DMSO) treatment, but CK2 inhibitors (DMAT, TBB, and DRB) significantly reduced pSer254 ENT1 levels in membrane fractions (GAPDH was used to normalize these ENT1 signals). The concentration of DRB used in the current study (100μM) has been shown to inhibit CK2 activities in rat acute hippocampal slices

(Lieberman and Mody 1999). We also used TBB at a concentration of 10μM; when tested against a panel of 33 other protein kinases, only CK2 was drastically inhibited (Sarno, Reddy et al. 2001). DMAT was used at a concentration of 5μM in our study, a concentration at which DMAT is highly selective for CK2 (Pagano, Meggio et al. 2004). These findings suggest that CK2 inhibition contributes to decreased phosphorylation of membrane-localized ENT1 in hippocampal brain tissue.

We also performed cell surface biotinylation assays in hippocampal brain slices, and quantified the levels of biotinylated ENT1 proteins phosphorylated at Ser254 using the pSer254 ENT1 antibody, A1R or A2AR antibody. As shown in Figure 3.3.3B, biotinylation assays revealed that the CK2 inhibitor - DRB produced a similar level of downregulation of pSer254 ENT1 (~50% reduced) compared to those obtained in membrane fractionation (Figure 3.3.3A). In contrast, the CK2 activator spermine (Hathaway and Traugh 1984) caused a significant upregulation of pSer254 ENT1 (Figure 3.3.3B). Since spermine and other polyamines are known to increase the function of NMDA receptors by binding to an extracellular region of the receptor (Ran, Miura et al. 2003), we incubated hippocampal slices with the NMDA receptor antagonist D-APV for 30min before applying spermine in order to rule out the possible confounding effect of NMDA receptor-mediated upregulation by spermine. However, spermine + D-APV-treated hippocampal slices showed similar levels of pSer254 ENT1 upregulation as in slices treated with spermine alone. Similarly, DRB reduced A1R surface expression but spermine increased the surface level of A1R. In contrast, DRB increased the A2AR surface levels but spermine decreased the surface levels of A2AR. Together, these data suggest a constitutive phosphorylation of ENT1 at the CK2 phosphorylation site Ser254, which can be reduced by CK2 inhibitors and enhanced by endogenous or exogenous polyamines. CK2 inhibition decreased the surface levels of pSer 254 ENT1 and A1R but increased the A2AR surface levels. In contrast, CK2 activation increased the surface levels of pSer 254 ENT1 and A1R but decreased the A2AR surface levels.

Figure 3.3.2



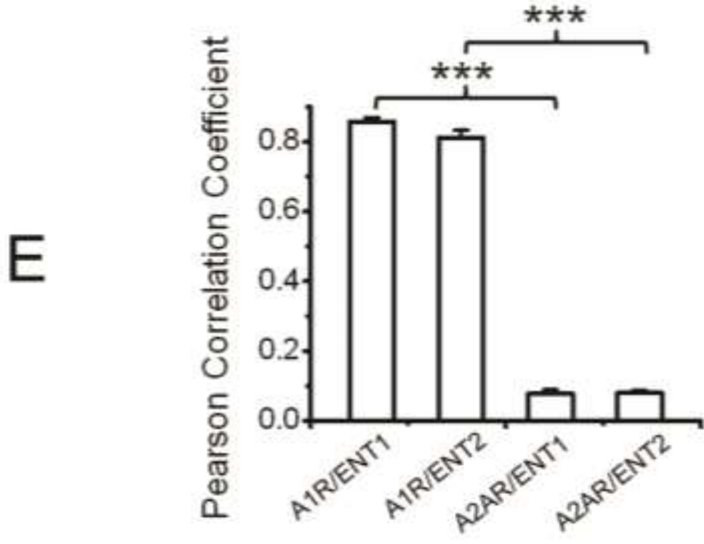


Figure 3.3.2 ENT1 and ENT2 colocalized with A1Rs in cultured hippocampal neurons. Confocal imaging microscopy reveals colocalization of ENT1 (A, staining with rabbit anti-ENT1 antibody) and ENT2 (B, staining with rabbit anti-ENT2 antibody) with adenosine A1 receptor (A1R, staining with goat anti-A1R antibody) in somas and dendrites of hippocampal neurons (colocalization indicated by yellow pixels in the merged channels or in inset panels). In contrast, the adenosine A2A receptor (A2AR, staining with mouse anti-A2AR antibody) did not colocalize with ENT1 (C) or ENT2 (D), as shown by the absence of yellow pixels in the merged and inset panels. MAP2 labeling with chicken anti-MAP2 antibody in A-C confirmed hippocampal neuron morphologies. Scale bars in A are 5μm (column 1) or 2μm (fifth column, inset panel), and apply to C-D. (E) Quantification of colocalization between ENTs and A1Rs by Pearson correlation coefficients revealed significant colocalization between A1Rs and ENT1/ENT2 (>0.8 arbitrary value), but not between A2ARs and ENT1/ENT2 (<0.1 arbitrary value). Values in E are means±SEM, N=14 each bar, ***P<0.001.

Protein	Accession Number	Sequence	Candidate CK2 site	NetPhosK Score
Rat ENT1	NP_113872	TKLD	T-248	0.54
		SEGE	S-254	0.67
Rat ENT2	NP_113926	TKAE	T-235	0.52

Table 3.1 Protein kinase CK2 phosphorylation consensus sequences in rat ENT1. Underlined amino acids correspond to serine (S) or threonine (T) residues that are phosphorylated by protein kinase CK2 (canonical sequence S/TXXD/E). Data was obtained from NetPhosK and NCBI GenPept.

Figure 3.3.3

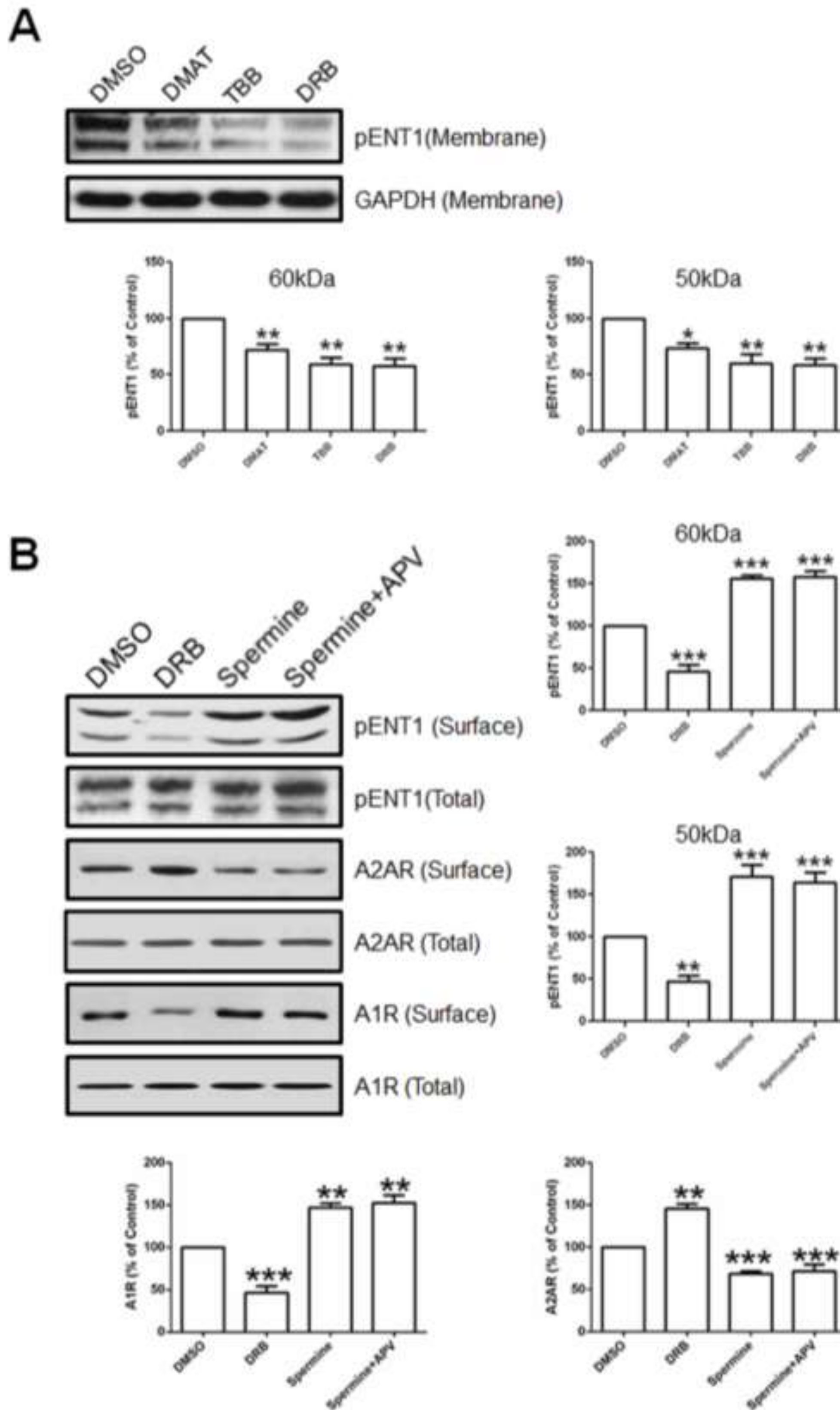
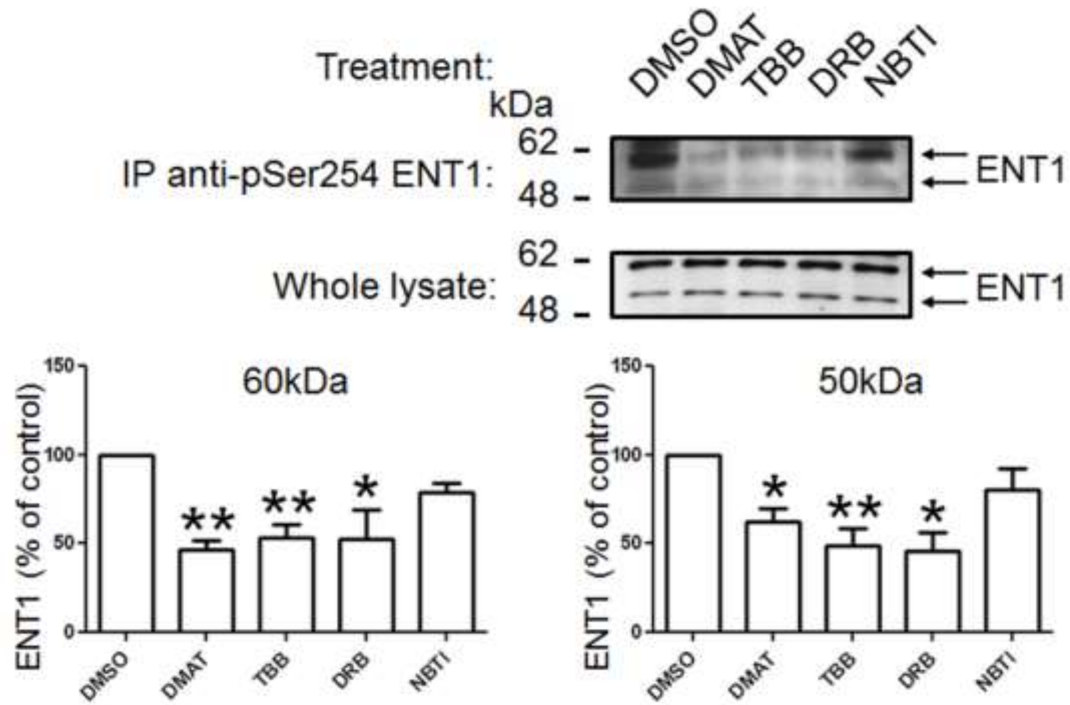


Figure 3.3.3 Inhibitors of CK2 decreased, activator of CK2 increased, phosphoSer254 ENT1 in rat hippocampal brain slices. (A) Hippocampal slices were pre-treated with the CK2 inhibitors DMAT (5μM), TBB (10μM), and DRB (100μM) for 1h before isolating membrane fractions. Immunoblots were probed with the phospho-specific antibody anti-pSer254 ENT1 antibody (Abgent) raised in rabbit. Protein bands were normalized to GAPDH using the same hippocampal membrane fractions. Summary bar charts show that both 50 kDa and 60 kDa pSer254 ENT1 signals in hippocampal membrane fractions were decreased by all CK2 inhibitors. (B) Hippocampal brain slices were pre-incubated with D-APV (50μM) for 30min before adding the CK2 agonist spermine (300μM). After 1h treatment with DRB or spermine, hippocampal slices were biotinylated (see Materials and Methods for details) and whole lysates were prepared. Biotinylated samples (top blot) and whole lysates (bottom blot) were immunoblotted with anti-pSer254 ENT1 antibody, anti-A2AR antibody or anti-A1R antibody. Surface protein levels were normalized to total protein levels, showing the changes in the protein levels in the different treatments. Summary bar charts show reduction in pSer254 ENT1 and A1R surface levels with CK2 inhibition, but upregulation in the presence of CK2 activation. In contrast, A2AR surface levels increased with CK2 inhibition, but decreased in the presence of CK2 activation. Average values in A and B are means±SEM, N=3 independent experiments, *$P<0.05$, **$P<0.01$, and ***$P<0.001$. (Acknowledgments: I thank Nicole L. Longmuir for contributing to the initial membrane fractionation biochemical studies that provided the basis for the membrane fraction data shown in Figure 3.3.3A.)

3.3.4 CK2 inhibition, but not ENT1 inhibition, decreased overall levels of pSer254 ENT1 and A1Rs in whole hippocampal brain homogenates

To confirm whether the downregulation of pSer254 ENT1 levels by CK2 inhibitors occurred specifically in membrane fractions or plasma membrane-associated (i.e., biotinylated) proteins, we studied the pSer254 ENT1 levels by using whole hippocampal brain slice homogenates and immunoprecipitation with the anti-pSer254 ENT1 antibody and probing the resulting western blots with rabbit polyclonal anti-ENT1 antibody (Millipore). We also determined whether the ENT1-selective inhibitor NBTI mimicked the effects of CK2 inhibition. As shown in Figure 3.3.4A (top blot, summarized in bar charts below), the levels of ENT1 containing the 50 kDa and 60 kDa pSer254 ENT1 immunoprecipitates were significantly decreased in the presence of the CK2 inhibitors (DMAT, TBB, and DRB) but were not altered in the presence of the ENT1 inhibitor NBTI. However, the total ENT1 levels in the whole lysate immunoblots remained constant throughout the different CK2 and ENT inhibitor treatments (Figure 3.3.4A, bottom blot), indicating that the decreased pSer254 ENT1 levels observed in the presence of CK2 inhibitors was not the result of protein degradation. Since we found that ENT1 (and ENT2) coimmunoprecipitated and colocalized with A1Rs (above), we determined whether the pSer254 ENT1 immunoprecipitates also contained A1Rs. After reprobing the blots in Figure 3.3.4A with the rabbit polyclonal A1R antibody (Sigma), the immunoblots also showed significant downregulation of A1Rs by CK2 inhibitors but not by ENT1 inhibitors (Figure 3.3.4B, top blot, summarized in bar chart below). As with by ENT1 in whole lysate immunoblots, the A1R levels in whole lysates were unchanged, suggesting the downregulation of A1Rs by CK2 inhibitors was not associated with significant protein degradation (Figure 3.3.4B, bottom blot). These results indicate that CK2 inhibition, but not ENT1 inhibition, contributes to decreased phosphorylation of ENT1 at Ser254, and that this CK2 phosphorylation site is an important determinant of ENT1 and A1R cell surface trafficking in hippocampal brain tissue.

Figure 3.3.4



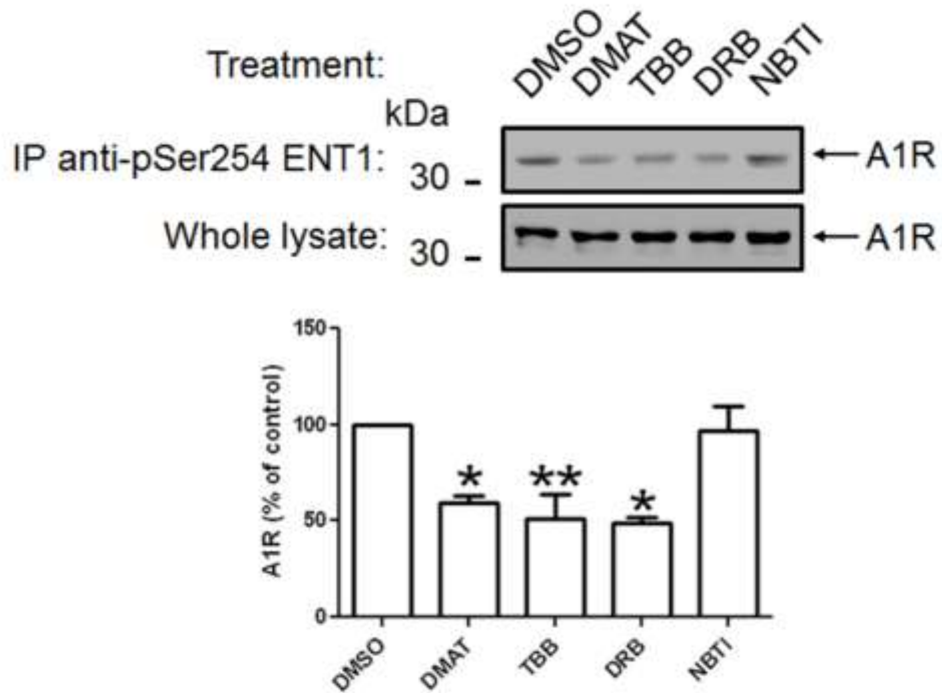


Figure 3.3.4 CK2, but not ENT1, inhibition decreased ENT1 phosphorylation in hippocampal brain slices. (A) Whole cell lysates prepared from hippocampal brain slices were used to immunoprecipitate ENT1 with anti-pSer254 ENT1 antibody (top blot). The resulting blot and the whole lysate blot (bottom blot) were immunoblotted with anti-ENT1 antibody. Summary bar charts show significant reduction in ENT1 phosphorylation in CK2 inhibitors DMAT (5μM), TBB (10μM), and DRB (100μM), but not in ENT1 inhibitor NBTI (100nM). (B) The same blots in A were re-probed with rabbit anti-A1R antibody, and summary bar chart shows CK2 inhibitors, but not ENT1 inhibitor, significantly reduced levels of A1Rs in pSer254 ENT1 immunoprecipitates. Average values are means±SEM, N=3 independent experiments, $^{*}P<0.05$ and $^{**}P<0.01$.

3.3.5 Both CK2 and ENT1 inhibition decreased ENT1 and ENT2 surface expression in primary cultured rat hippocampal neurons

To determine whether the changes in ENT1 phosphorylation levels described above are occurring specifically in hippocampal neurons, and whether the surface expression of ENT1 and ENT2 are depend on CK2 activity or functional ENTs, we used primary cultures of hippocampal neurons and biotinylation assays to assess the relative surface densities of ENT1 and ENT2 in the absence or presence of both CK2 and ENT1 inhibitors. As shown in immunoblots in Figure 3.3.5A (top panel), the CK2 inhibitors TBB, DMAT, and DRB, and ENT1 inhibitor (NBTI) significantly decreased the surface expression of the 50 kDa and 60 kDa ENT1 bands in cultured hippocampal neurons (see summary bar charts in Figure 3.3.5A, bottom). However, pre-incubation with the A1R antagonist DPCPX prevented downregulation of ENT1 surface expression by CK2 and ENT1 inhibitors (Figure 3.3.5A, top panel, lanes 6-7; summary bar charts in Figure 3.3.5A, bottom). When blots containing biotinylated proteins were reprobed with an anti-beta actin antibody, no beta actin signal was observed in these blots, indicating that the biotinylated proteins were uncontaminated with cytosolic proteins (Figure 3.3.5A, third panel), however, beta actin was detected in blots containing the whole cell lysates (Figure 3.3.5A, fourth panel). More importantly, the whole cell lysate immunoblots showed a constant level of ENT1 across all treatments (Figure 3.3.5A, second panel), suggesting that the downregulation of ENT1 surface expression by CK2 and ENT1 inhibitors was not caused by increased protein degradation. Similar to ENT1, ENT2 surface expression was also reduced by approximately 50% from CK2 and ENT1 inhibitors (Figure 3.3.5B, top blot), and DPCPX prevented these inhibitory effects (summary bar charts in Figure 3.3.5B, bottom). Together, these results indicate that inhibitions of CK2 and ENT1 functions in hippocampal neurons promotes decreased surface expression of ENT1 and ENT2, which requires functional adenosine A1Rs.

Figure 3.3.5

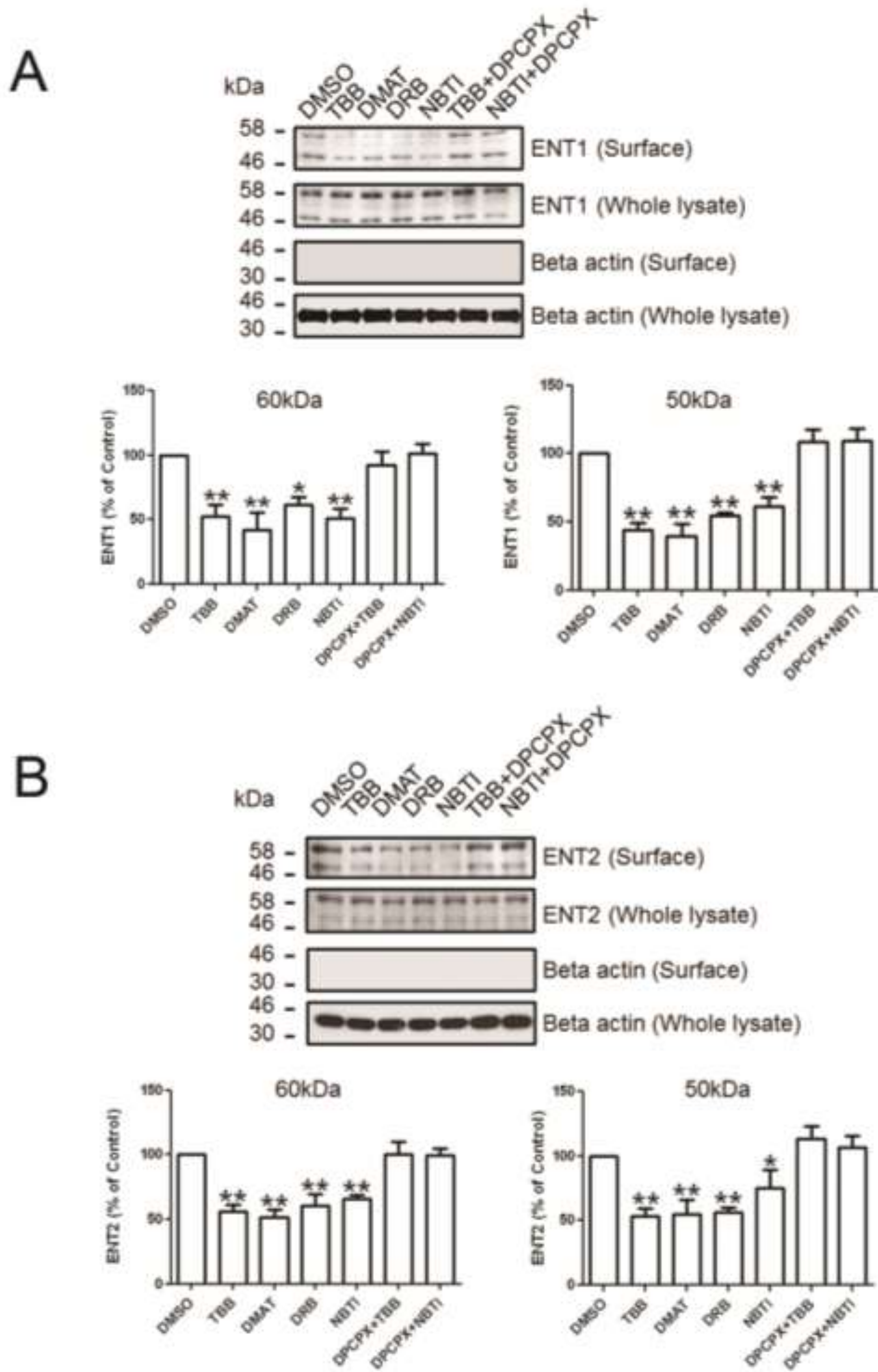


Figure 3.3.5 Both CK2 and ENT1 inhibition decreased ENT1 surface expression in primary cultures of hippocampal neurons. (A) Surface labeling of proteins was performed using biotinylation assays and whole lysates from cultured hippocampal neurons. *Top panel*. Biotinylated proteins (top blot) show significant downregulation of ENT1 surface expression (anti-ENT1 from Millipore) in presence of both CK2 and ENT1 inhibitors, an effect prevented by pretreatment with A1R antagonist DPCPX (100nM). No significant protein degradation (second blot) or contamination with cytosolic proteins such as beta actin (third blot) accompanied this effect. Beta actin signals were present in whole lysate blots (fourth blot). *Bottom panel*. Summary bar charts showing significant downregulation of surface-expressed ENT1 in presence of CK2 and ENT1 inhibitors, and DPCPX prevented these effects. (B) Similar experiments were performed as in A, except blots were probed with anti-ENT2 antibody. Summary bar charts are also showing significant downregulation of ENT2 surface expression by CK2 and ENT1 inhibitors, but not when hippocampal neurons were pre-treated with DPCPX. Average values for experiments shown in A and B are means±SEM, N=3 independent experiments, *P<0.05 and **P<0.01.

3.3.6 CK2 and ENT inhibition enhances hypoxia-mediated downregulation of ENT and A1R surface expression

Since hypoxia and ischemia are known to cause increased adenosine tone and increased A1R activation (Dunwiddie and Masino, 2001; Fowler, 1993; Frenguelli et al., 2003), along with the results presented above we hypothesized that CK2 and ENT inhibition leads to pronounced downregulation of ENTs and adenosine receptors during hypoxia. Using surface biotinylation assays and hippocampal brain tissue subjected to a 20min hypoxic insult, we confirmed that CK2 and ENT inhibitors enhanced hypoxia-mediated reductions in the abundance of surface-expressed ENT1, ENT2, A1Rs, as well as hypoxia-mediated increase in the amount of surface-expressed A2ARs. As shown in Figure 3.3.6A, hypoxia reduced the surface expression of A1Rs by ~20% compared to normoxia, but increased the surface expression of A2ARs by ~50%. The CK2 inhibitor DRB, the ENT1-selective inhibitor NBTI and the ENT1/ENT2 inhibitor DPY all potentiated the hypoxia-mediated decrease in A1R surface expression (Figure 3.3.6A, summary bar chart), producing ~40-60% reduction in A1R levels during hypoxia. Similarly, the surface expression of ENT1 (both 50kDa and 60 kDa bands) was decreased by hypoxia, and this reduction was potentiated by CK2 and ENT inhibitors (by ~70-80% vs. 30% in hypoxia alone; see Figure 3.3.6B, summary bar chart for ENT1). Similar to the influence on ENT1 and A1Rs, the CK2 and ENT inhibitors also potentiated the downregulation of ENT2 surface expression during hypoxia (Figure 3.3.6B, see summary bar chart for ENT2). Additionally, both ENT1 and CK2 inhibitors decreased the surface levels of A2ARs (Figure 3.3.6B, see summary bar chart for A2ARs).

In contrast to the attenuation of surface expression, the levels of A1R, A2AR, ENT1, and ENT2 remained constant in whole hippocampal lysates (Figure 3.3.6), suggesting that protein degradation did not significantly contribute to these changes in surface expression during a 20min hypoxic insult with or without CK2 and ENT inhibitors. Taken together, the results indicate that A1Rs, A2ARs, and ENTs are functionally linked via extracellular adenosine levels and by intracellular CK2 activities.

Figure 3.3.6

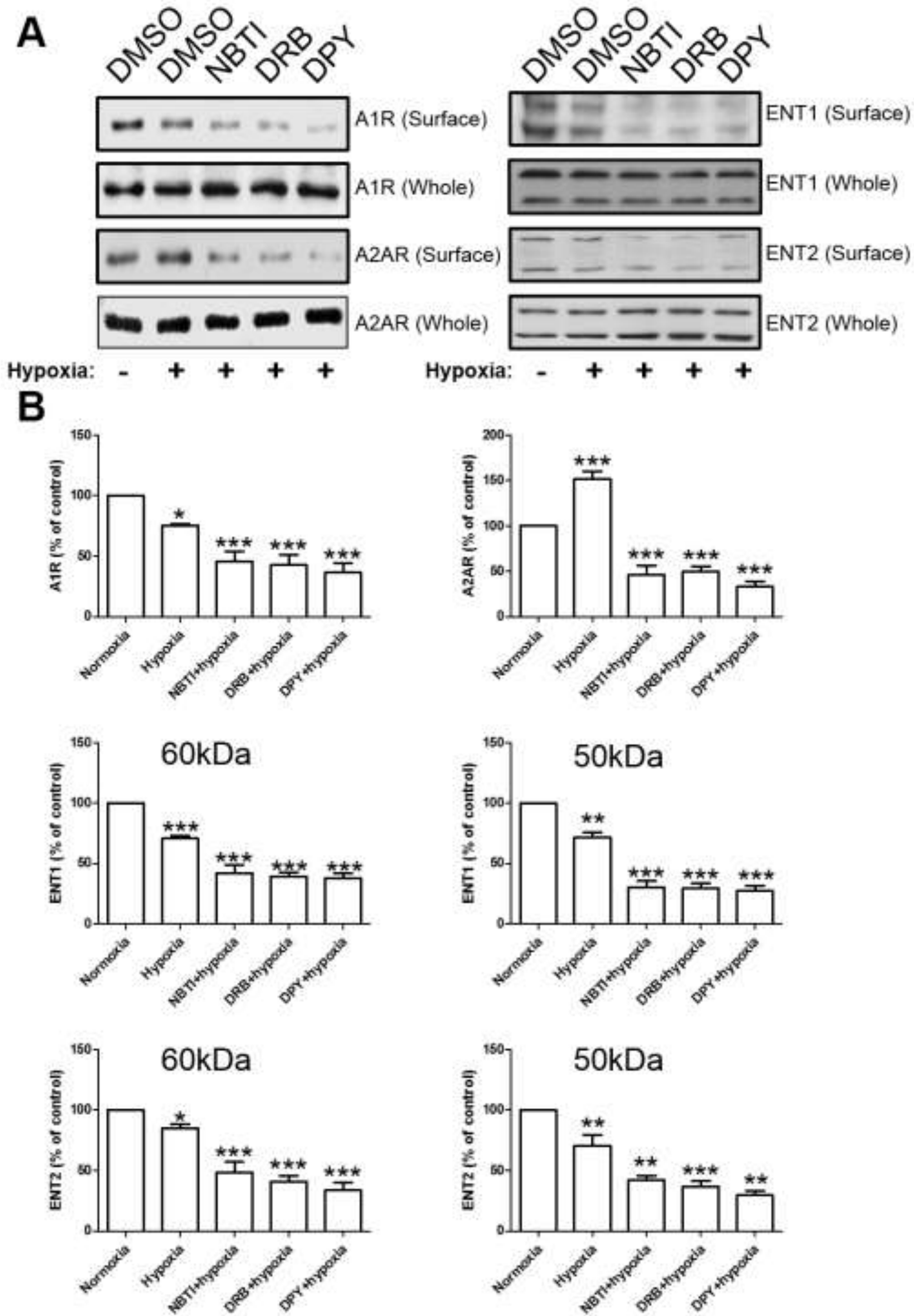
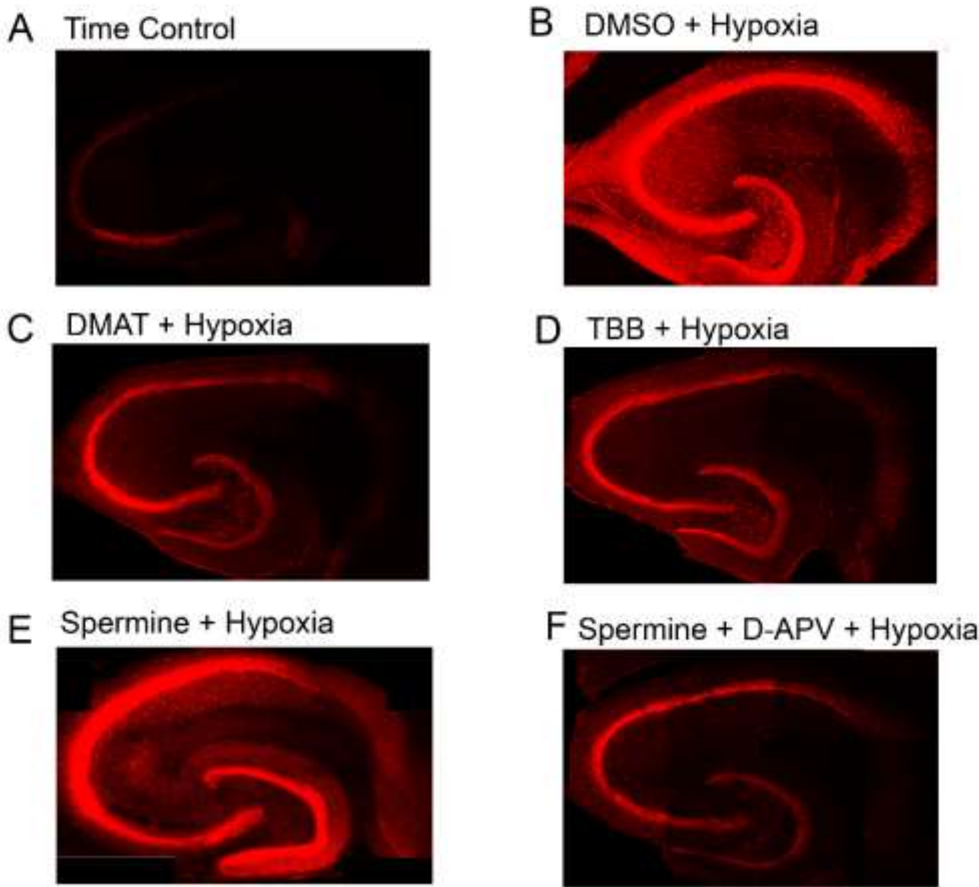


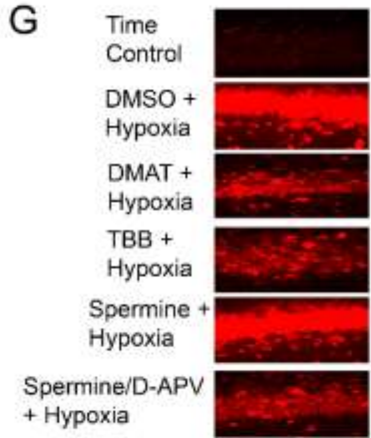
Figure 3.3.6 CK2 and ENT inhibitors induced downregulation of A1R, A2AR, ENT1, and ENT2 surface expression in hippocampal brain slices after hypoxic stimulation. Hippocampal brain slices were pre-treated with DMSO (control), ENT inhibitors NBTI (100nM) and DPY (10μM), or CK2 inhibitor DRB (100μM) for 1h before a 20 min hypoxic insult. After hypoxia, brain slices were immediately subjected to biotinylation to assess the relative abundance of A1R, A2AR, ENT1, and ENT2 on the surface of hippocampal tissue. (A) The CK2 inhibitor DRB, ENT1 inhibitor NBTI, and ENT1/ENT2 inhibitor DPY enhanced hypoxia-mediated downregulation of A1R surface expression. The inhibitors also decreased the hypoxia-mediated increase of A2AR surface expression that was seen in control. The inhibitors enhanced the hypoxia-mediated downregulation of ENT1 and caused a further enhancement of hypoxia-mediated reduction of ENT2 surface expression. (B) Densitometry values for the whole lysate blots were used to normalize all surface (biotinylated) values. Whole lysate signals did not differ between treatments. Values in summary bar charts are means±SEM from N=4 independent experiments, *p<0.05, **P<0.01, and ***p<0.001).

3.3.7 CK2 inhibition provided neuroprotection to hippocampal slices after 20min hypoxia

The role of CK2 as a potential neuroprotective protein has been explored (Kim, Jung et al. 2009), but the direct effect of CK2 inhibition or activation in neuroprotection or neurodegeneration in hypoxia or ischemia has not been well established. To test the effect of CK2 in hypoxia-induced neurodegeneration, hippocampal slices were pre-treated with DMAT or TBB, which are both CK2 inhibitors, or with spermine, which is a CK2 activator, or, due to the ability for spermine to also activate NMDA receptors by direct interaction, we also treated slices with spermine + D-APV, an NMDAR inhibitor. Following a 20min hypoxic insult, slices were reintroduced to normoxic conditions and incubated for 3h prior to fixation. In the final hour of incubation, propidium iodide (PI) was added to label dead cells for neurodegeneration. The results showed that hypoxia alone induced significant neurodegeneration (Figure 3.3.7B), whereas slices treated with DMAT and TBB showed significantly less neurodegeneration than hypoxia alone (Figure 3.3.7C-D) Time control slices showed significantly less neurodegeneration than hypoxia control (DMSO), and densitometry analysis was performed with densitometry values normalized to time control (100%). DMAT and TBB treatment prior to hypoxia were shown to be neuroprotective, while spermine did not prevent hypoxia-induced neurodegeneration (Figure 3.3.7E). Spermine with the NMDAR inhibitor D-APV was also neuroprotective, indicating that CK2 activation may contribute to neurodegeneration through increasing NMDAR activity. These data suggest that inhibition of CK2 activity, or reduced CK2 activation, affords neuroprotection to hippocampal neurons.

Figure 3.3.7





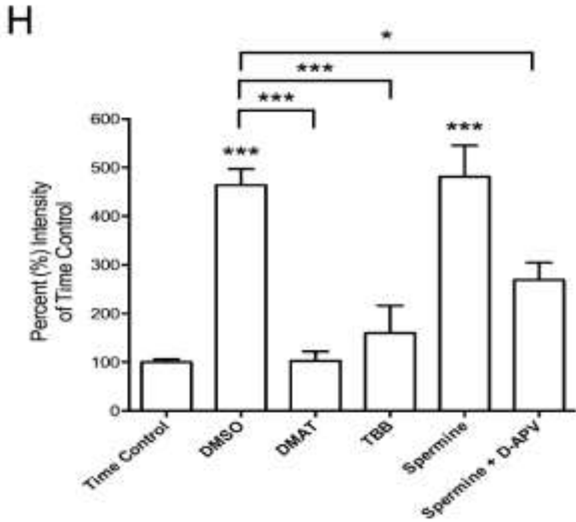


Figure 3.3.7 CK2 inhibitor treatment reduced neurodegeneration caused by a 20min hypoxic insult in hippocampal slices. (A) Representative propidium iodide staining images of time control hippocampal slices, which were not subjected to any drug treatments or hypoxic conditions. There is very little neurodegeneration seen in these slices, and all treated slices were normalized to the time control values (100%). (B) Slices treated with DMSO with hypoxia showed significant neurodegeneration approximately 450% of time control values. (C) Slices treated with DMAT showed significantly less neurodegeneration compared to DMSO. (D) Representative slice treated with the CK2 inhibitor TBB. (E) Representative images showing increased neurodegeneration in hippocampal slices treated with spermine alone. (F) Representative hippocampal slice showing treatment with spermine and D-APV. (G) Representative propidium iodide staining in CA1 region. (H) Summary bar graph showing values of average intensity normalized to time control values (100%). Average values are shown as mean ± SEM with arbitrary units, n=4 independent experiments, 5 rats per experiment. Significance values * $P<0.05$ and *** $P<0.001$. (Acknowledgments: I thank Jocelyn Stockwell who performed the staining and acquired the confocal microscopy imaging shown in Figure 3.3.7)

3.4 Discussion

The equilibrative nucleoside transporters ENT1 and ENT2 are expressed ubiquitously in mammalian cells, and are distributed throughout the human central nervous system, including the hippocampus (Jennings 2001). Little is known about the regulation of these transporters, but protein kinase modifiers, such as inhibitors of PKC, have been shown to modulate the activity of these transporters in a variety of non-neuronal cell types (Coe, Zhang et al. 2002; Kong, Engel et al. 2004). ENT1 and ENT2 have a large intracellular loop joining the transmembrane domains 6 and 7, where putative CK2 phosphorylation consensus sequences are located (see Table 3.1) (Stolk, Cooper et al. 2005). These phosphorylation sites suggest that the ENT activity and/or its membrane expression can be susceptible to modulation by CK2. In the present study, we found that CK2 inhibitors (TBB, DMAT and DRB) decreased, while the CK2 activator spermine increased, phosphorylation of ENT1 proteins at Ser254 in rat hippocampal tissue. Moreover, CK2 inhibition significantly downregulated the ENT surface expression in rat hippocampal

slices and primary cultured hippocampal neurons. In contrast, direct ENT1 inhibition with NBTI did not alter the levels of pSer254 ENT1, but did cause a significant A1R-dependent downregulation of ENT1 surface expression. Furthermore, both CK2 and ENT inhibitors enhanced hypoxia-mediated downregulation of ENTs and A1Rs and reduced hypoxia-induced synaptic depression. Together with biochemical and confocal imaging evidence supporting a physical interaction between ENTs and A1Rs, these results indicate that CK2-mediated phosphorylation of ENTs contributes to the regulation of extracellular adenosine levels and to the surface trafficking of ENTs and A1Rs. This represents a potentially important mechanism that plays a role in hypoxic/ischemic neuronal damage.

In other studies, inhibition of the CK2 activity in human U2-OS cells via transfection with a catalytically inactive CK2α' subunit increased ENT1 function and expression (Bone, Robillard et al. 2007), which is opposite to what we found in the present study using different CK2 inhibitors. However, it is possible that the modification of the CK2 activity can alter ENT expression and function in a cell- or tissue-specific manner as well as in different experimental preparations. Our data suggest that ENT1 and ENT2 expression in hippocampal membrane is constitutively regulated by the CK2 activity. These findings are in agreement with those found by Bone et al., whereby TBB-treated cells reduced the number of functional ENT1 proteins at the level of the plasma membrane, a response that was mimicked by deletion of Ser-254 from mENT1 (Bone, Robillard et al. 2007). Although it is not recognized in rat or human tissue, a splice variant of ENT1 has been identified in murine tissue (Kiss, Farah et al. 2000; Handa, Choi et al. 2001). The shorter splice variant (mENT1a), which lacks two amino acids (Lys255 and Gly256) and has an arginine in place of a serine at position 254, has greater expression in brain tissue than the larger variant (mENT1b) (Bone, Robillard et al. 2007). It was also shown that the number of mENT1b transporters operating at the level of the plasma membrane are downregulated by the CK2 inhibition, while mENT1a is not (Bone, Robillard et al. 2007). In our present study, we found that the 60 kDa ENT1 isoform was more prevalent than the 50 kDa ENT1 isoform in whole cell lysates from rat hippocampal brain tissue and hippocampal neurons. It is possible that the 50 kDa ENT1 proteins, which are expressed at lower levels, may reflect the presence of a yet to be identified rat ENT1 splice variant with lower susceptibility to regulation by CK2 phosphorylation. However, this seems unlikely since

both 50 kDa and 60 kDa ENT1 bands were shown to be equally susceptible to CK2 inhibitors and both ENT1 bands can be immunoprecipitated with the pSer254 ENT1 antibody. Alternatively, as previously suggested by other studies (Crawford, Patel et al. 1998; Coe, Zhang et al. 2002; Reyes, Naydenova et al. 2010), differential glycosylation of ENT1 can account for the different sizes observed for ENT1. Nevertheless, our results indicate that both 50 and 60 kDa ENT1 proteins are constitutively phosphorylated at Ser-254, a known target for CK2 (Kiss, Farah et al. 2000; Handa, Choi et al. 2001), and that CK2 phosphorylation regulates increased surface trafficking of both ENT1 and ENT2 in hippocampal neurons and hippocampal brain slices.

Since ENTs are known to regulate extracellular levels of adenosine during hypoxia and ischemia (Dunwiddie and Masino 2001; Frenguelli, Wigmore et al. 2007; Chu, Xiong et al. 2013), the possibility of a protein signaling complex involving ENTs and adenosine receptors would certainly allow the rapid transduction of extracellular adenosine elevations via A1R or A2AR activation. Using coimmunoprecipitation and confocal imaging studies, we determined that ENTs are physically associated with A1Rs but not with A2ARs, and selective A1R stimulation downregulates ENT1 surface expression. Moreover, we observed a similar pattern of downregulation in A1R and ENT surface expression when hippocampal brain slices were pretreated with ENT or CK2 inhibitors before hypoxic stimulations. These data indicate that the ENT-A1R protein-protein interactions can provide an important link between adenosine transport and intracellular signaling pathways activated by A1Rs that can contribute to the downregulation of ENT and A1R surface expression. However, future studies are needed to further characterize the nature of A1R and ENT interactions (i.e., either direct or indirect), and to identify the CK2-regulated signaling pathways involved in ENT and A1R downregulation.

CK2 is known to be widely expressed in the hippocampus (Willingham 1997; Blanquet 1998; Blanquet 2000), but whether CK2-mediated regulation of ENT surface expression help protect hippocampal neurons against ischemic cell degeneration remains unclear. Several studies have shown that CK2 activity was decreased in vulnerable brain regions, including the hippocampal CA1 region and the striatum, and it aggravated brain damage and cell death after ischemic injury (Hu and Wieloch 1993; Kim, Jung et al. 2009). In

contrast, CK2 activity increased in brain regions that are resistant to ischemic damage (Hu and Wieloch 1993). Moreover, the activators of CK2, spermine and spermidine, were shown to protect neural cells against ischemic damage (Gilad and Gilad 1991). Other studies using ENT blockers or CK2 inhibitors (Dunwiddie and Diao 1994; Lieberman and Mody 1999; Pearson, Nuritova et al. 2001; Ackley, Governo et al. 2003; Frenguelli, Wigmore et al. 2007) have demonstrated an inhibition of hippocampal synaptic transmission, but whether this has the potential to promote neuroprotection during ischemic brain damage is not established. However, adenosine transport inhibitors have been reported to be neuroprotective (Noji, Karasawa et al. 2004), likely resulting from their ability to dramatically increase adenosine tone during cerebral ischemia. In the present study, we observed that hypoxia decreased the surface expression of ENT1, ENT2 and A1R in rat hippocampus; this inhibitory effect was further enhanced by preincubation of hippocampal slices with CK2 or ENT inhibitors. This downregulation in ENT surface expression can contribute to the well documented reports of extracellular adenosine elevation during hypoxia/ischemia and subsequent A1R-mediated synaptic depression (Dunwiddie and Masino 2001; Frenguelli, Wigmore et al. 2007; Zhang, Xiong et al. 2011). Consistent with our observations, other studies of human umbilical vein endothelium exposed to a hypoxic environment showed a reduction in hENT1 function, ENT1 protein and mRNA levels, but an increase in extracellular adenosine concentration (Casanello, Torres et al. 2005). Moreover, an intriguing observation is that increasing hENT1 expression in mice led to increased ischemic brain damage that was prevented by caffeine (A1R and A2AR antagonist) administration (Soylu, Zhang et al. 2012; Chu, Xiong et al. 2013). However, future studies are needed to establish whether CK2 inhibition and subsequent downregulation of A1Rs and ENTs in hippocampus results in neuroprotection or increased neuronal damage after hypoxia/cerebral ischemia.

ENTs are the main adenosine transporters and control adenosine levels in the brain (Dunwiddie and Masino 2001). It was reported that extracellular adenosine levels were increased in the striatum of ENT1−/− knockout mice (Kim, Karpyak et al. 2011; Nam, Lee et al. 2011). Inhibition of ENT1 also increased extracellular adenosine levels (Nagy, Diamond et al. 1990). In addition, inhibition of adenosine uptake by the ENT inhibitors NBTI and DPY increased adenosine levels in the olfactory bulb (Sanderson and

Scholfield 1986). The selective A1R antagonist, DPCPX prevented the A1R-induced synaptic inhibition, allowing an increase in synaptic transmission (Ciruela, Casado et al. 2006). Since the A1R has a higher binding affinity to adenosine (Kd ~70nM) than A2AR (Kd ~150 nM) (Dunwiddie and Masino 2001), increased adenosine levels by ENT inhibition may predominantly affect A1R-mediated synaptic depression, but this enhanced adenosine tone could also activate A2ARs which mediate excitatory actions that could lead to neurodegeneration. Here, we showed that CK2 inhibition dramatically reduced neuronal death after hypoxia-reperfusion injury, and the converse was true with CK2 activation which enhanced neuronal death even in presence of NMDAR antagonist D-APV. Our present results would suggest that CK2 inhibition, which leads to downregulation of ENTs and A1Rs, promotes less neuronal death with subsequent hypoxic/ischemic insult. This is consistent with the finding by Soylu and colleagues (Soylu, Zhang et al. 2012). Our findings showed that acute CK2 inhibition decreased ENT and A1R surface expression, suggesting that the reduced availability of surface levels of ENTs and A1Rs is important for subsequent response to hypoxic/ischemic insults to the brain.

In conclusion, our study provides novel evidence that ENTs are physically coupled with A1Rs, and this coupling facilitates the CK2-mediated cell surface trafficking of ENT1, ENT2 and A1R in the rat hippocampus. We also showed that during hypoxia the CK2 and ENT inhibitors caused a more marked downregulation of ENT1, ENT2 and A1R surface localization in hippocampal tissue. These data suggests that the inhibition of CK2 and ENT functions results in the accumulation of extracellular adenosine that subsequently acts on postsynaptic A1Rs to cause further downregulation of ENT and A1Rs. These CK2-mediated changes in ENT and A1R surface expression represent an important mechanism for cerebral ischemic damage, but future studies are needed to determine whether activations of CK2 and consequently increased surface trafficking of ENTs are important for neuroprotection.

CHAPTER 4

Adenosine A1 receptor-mediated endocytosis of AMPARs contributes to impairments in long-term potentiation (LTP) in the middle-aged rat hippocampus

Zhicheng Chen and Francisco S. Cayabyab

Abstract

There is a high prevalence of neurological disorders in the elderly, such as stroke, Alzheimer's disease, and Parkinson's disease, and these neurodegenerative diseases are often associated with cognitive deficits. In addition, the aged brains of humans and animals have been shown to have enhanced extracellular levels of adenosine. Previously in Chapter 2, I reported that activation of the adenosine A1 receptors (A1Rs) induces internalization of AMPARs in rat hippocampus. In the present study, I used biochemical and electrophysiological techniques to test the hypothesis that in rat hippocampus the expected enhancement of adenosinergic signaling in aged brains produces a reduction in the surface levels of AMPARs which could lead to attenuation of synaptic plasticity. I found that the surface levels of AMPARs decreased in the aged hippocampus. Using a well-established protocol to test for changes in synaptic plasticity, I recorded fEPSPs and compared chemically-induced long-term potentiation (cLTP) in the hippocampus of young and old rats. I found that middle aged brains (7-12 months old) compared to juvenile brains (1 month old) showed significant impairments in cLTP, suggesting that aging impaired synaptic plasticity. The surface levels of AMPARs were evaluated before and after cLTP in young and middle aged hippocampus. The results showed that cLTP significantly increased the surface levels of AMPARs (both GluA1 and GluA2 subunits) in young hippocampal slices, whereas only a modest increase of surface AMPARs was observed after cLTP in older hippocampus. As AMPAR trafficking could go through the clathrin-mediated endocytosis pathway, I then determined that blocking this pathway with Tat-GluA2-3Y peptide or Dynasore similarly enhanced the cLTP-induced increases in AMPAR surface expression. I also found that A1Rs were decreased while A2ARs were increased in older brains. These results indicate that increased adenosinergic signaling in aged brains leads to changes in adenosine receptor density, which contributes to increased clathrin-mediated endocytosis of AMPARs and impaired synaptic plasticity.

4.1 Introduction

Previously, we reported (Chen et al., 2014, see also Chapter 2) that prolonged A1R stimulation leads to clathrin-mediated endocytosis of GluA2 and GluA1 AMPARs, which we suggested contributes to the adenosine-induced persistent synaptic depression (APSD) and neurodegeneration in juvenile (17-30 days old) hippocampal brain slices. Here, we studied the potential contribution of adenosine-induced downregulation of these AMPARs to changes in synaptic plasticity in middle-aged rats. Elevated extracellular adenosine levels were reported in hippocampal slices from 24-month-old (old) as well as 12-month-old (middle-aged) rats (Sperlagh, Zsilla et al. 1997; Murillo-Rodriguez, Blanco-Centurion et al. 2004). It has been suggested that this increase in extracellular adenosine levels in aged rats is related to increased activity of 5'-nucleotidases, which break down ATP to adenosine (Zimmermann 1996; Cunha, Almeida et al. 2001; Murillo-Rodriguez, Blanco-Centurion et al. 2004). Adenosine is an endogenous neuromodulator which functions by inhibiting the release of transmitters such as glutamate, and interacting with other transmitter systems (Fredholm, Chen et al. 2005).

Aging is associated with cognitive deterioration (Ritchie, Carriere et al. 2007). Memory losses were found in aged rats (about 24 months old) (Granger, Deadwyler et al. 1996). LTP impairment was also described in aged rats (Barnes 1979; Barnes and McNaughton 1985; Deupree, Turner et al. 1991; Shankar, Teyler et al. 1998). In addition, the memory decline and LTP impairment were found in middle-aged rats (about 12 months old) (Barnes 1979; Barnes and McNaughton 1985; Deupree, Bradley et al. 1993; Granger, Deadwyler et al. 1996; Oler and Markus 1998; Ward, Oler et al. 1999; Meneses, Manuel-Apolinar et al. 2004). To our knowledge, there are only a few studies that reported the mechanism of LTP impairment in middle-aged rats (Rex, Kramar et al. 2005). It is widely accepted that adenosine plays an important role in synaptic plasticity in the hippocampus (Ribeiro 1995; Costenla, De MendonÃ§a et al. 1999; Lu, Zhou et al. 2010). Here, I investigated the role of adenosine signaling in regulating LTP induction and maintenance in young and middle-aged rat hippocampus. The evaluation of electrical synapses and dendritic spines following LTP induced via tetanic stimulation, presents the difficulty that not all synapses examined are necessarily activated. It has also been reported that LTP induced by high frequency stimulation can be reduced or depotentiated

by endogenous adenosine signaling in young and aging brains (de Mendonca and Ribeiro 1994; de Mendonca, Almeida et al. 1997; de Mendonca and Ribeiro 2000). To overcome this limitation, and to ensure that a very large proportion of the synapses and spines examined have been potentiated, I used chemical induction of LTP (cLTP) in acute hippocampal slices of adult rat by the addition of 50 μM forskolin and 0.1 μM rolipram in ACSF followed by 1h recoding of fEPSP in normal ACSF(see Method 4.2). This method of LTP induction using forskolin and rolipram was previously described by several laboratories (Otmakhov, Khibnik et al. 2004; Oh, Derkach et al. 2006; Kim, Futai et al. 2007; Schapitz, Behrend et al. 2010).

Since we have recently reported (Chen et al., 2014) that stimulation of A1Rs induced internalization of AMPARs, I hypothesized that enhanced adenosinergic signaling in aged brains may underlie deficits in LTP. AMPARs are tetramers composed of the receptor subunits GluA1-4, which together form a pore complex (Hollmann and Heinemann 1994). This complex is known to play a critical role in LTP, as shown by experiments with GluA1-/- mice that fail to show induction of LTP (Zamanillo, Sprengel et al. 1999). Similarly, previous reports indicate that aged rats have impaired LTP formation, supporting the importance of AMPARs in memory formation (Shankar, Teyler et al. 1998; Rex, Kramar et al. 2005). In addition, activation of adenosine receptors modulates synaptic plasticity (LTP) differently in young, middle-aged and aged rats (Costenla, Diogenes et al. 2011). LTP magnitude is more affected by inhibition of A1R in young rats than in aged rats (Costenla, Diogenes et al. 2011). A decreased efficiency of A1R to regulate synaptic transmission was also seen in aged rats (Sebastiao, Cunha et al. 2000). However, whether these changes in levels of LTP induction are related to altered levels of AMPARs and adenosine receptors has not yet been fully elucidated.

The function of A1R and A2AR during aging has previously been investigated (Cunha 2005; Rodrigues, Canas et al. 2008). A reduction in DPCPX binding ability to A1R was shown in the hippocampus of old versus young rats (Sperlagh, Zsilla et al. 1997). The level of A1R decreased in 12-month-old rats compared to 2-month-old rats. These results are consistent with the finding that reduced expression of A1R in the plasma membrane have also been confirmed in aged rats (Cunha, Dolores Constantino et al. 2001). High adenosine levels apparently induced desensitization and downregulation of

A1R in older brains (Leon, Albasanz et al. 2005), a finding that was confirmed by a later study (Sebastiao, Cunha et al. 2000). Therefore, we predicted that elevated adenosine levels during aging could alter not only the surface levels of adenosine receptors but also AMPARs.

Recently, we found a novel physical interaction between A1Rs and AMPARs which represents an important mechanism that influences the surface localization of adenosine receptors and AMPARs (Chen et al., 2014, see also Chapter 2). Since previous studies suggest that adenosine tone increases in aged brains (Cunha, Constantino et al. 1995; Rebola, Sebastiao et al. 2003), I therefore investigated the possibility that elevated adenosine in older hippocampus would promote a reduction in AMPAR surface expression, which could lead to significant impairment in LTP in aged brains. Therefore, the purpose of this study was to assess age-related changes in surface-expressed AMPARs and the impact of these changes on chemical LTP induction. Since the level of physical and functional association of adenosine receptors and AMPARs is not yet known in aged rats, I also investigated the possible altered association of adenosine receptors and AMPARs in middle-aged rats, and determined whether this alteration includes clathrin-mediated endocytosis of AMPARs and underlies impairments in synaptic plasticity as measured with the cLTP protocol.

4.2 Detailed Methods

4.2.1 Hippocampal slice preparation and treatments

1, 3, 6-7 and 10-12 month-old male Sprague-Dawley rats were anaesthetized with halothane and rapidly decapitated according to protocols approved by the University Committee of Animal Care and Supply at the University of Saskatchewan. Brains were extracted and placed into disection medium. Hippocampal slices (400 μm thick) were cut with a slicer (VT1000S, Leica, Nussloch, Germany) and maintained for 60-90 minutes in artificial CSF (ACSF) solutions (recipes for ACSF and dissection medium recording conditions were described previously (Chen et al., 2014; Brust et al., 2007; Brust et al., 2006) before performing biochemical experiments or electrophysiological recordings.

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4.2.2 Electrophysiology

To obtain LTP, field excitatory postsynaptic potentials (fEPSPs) were evoked by application of the forskolin (50 μM)/rolipram (0.1 μM) protocol, as previously described (Kim et al., 2007; Oh et al., 2006; Otmakhov et al., 2004; Schapitz et al., 2010). To assess cLTP, a 20 min recording of fEPSPs was obtained to ensure presence of stable baseline, prior to the 10 min recordings of fEPSPs in the presence of 50 μM of Forskolin and 0.1 μM of Rolipram (no $MgCl_2$). This was followed by a 1 hour recording of fEPSPs in normal ACSF (with normal Ca^{2+} and Mg^{2+} concentrations, see recipe in Chen et al., 2014; Brust et al., 2007; Brust et al., 2006). fEPSP signals were amplified 1000 times with an AC amplifier, band-pass filtered at 0.1-100 Hz, digitized at 10 kHz using a Digidata 1320A interface board (Axon Instruments, Foster City, CA), and transferred to a computer for analysis. Data were analyzed using Clampfit 10.2 (Axon Instruments). Baseline synaptic responses were established by evoking fEPSPs every 30 s for at least 20 min. The fEPSP slope was normalized to the mean of the 5 sweeps (2.5 min) immediately preceding drug perfusion. The mean normalized fEPSP slope was plotted as a function of time with error bars representing the standard error of the mean (SEM). Sample traces are the average of 5 sweeps from a recording that was included in the plot of the mean normalized fEPSP slope. All bar graphs show the mean normalized percent potentiation from baseline ± SEM. Statistical significance was assessed using one-way analysis of variance with Student-Neuman-Keuls post hoc test.

4.2.3 Biochemistry

Hippocampal slices were treated with normal Ringer-solution (1 mM $MgCl_2$, 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 33 mM (D)-glucose, 25 mM HEPES, pH 7.3) prior to cLTP (10 min in Ringer-solution (Mg^{2+}-free) supplemented with 50 μM forskolin and 0.1 μM rolipram (TOCRIS)). The solvent DMSO (Sigma, 1:1000) was used as a control. After washout for one hour in normal Ringer-solution, a biotinylation assay was performed as described below. After treatments, slices were cooled to 4 °C (20-30 min), and then washed with ice-cold ACSF before biotinylation. Slices were then incubated with 1 mg/ml NHS-SS-Biotin (Pierce) at 4 °C. The reaction was stopped by addition of a glycine buffer

(192 mM glycine, 25 mM Tris pH 8.3). Slices were then homogenized into lysis buffer that contained 50mmol/L Tris (pH8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF; protease inhibitors: 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, 2 mmol/L Na_3VO_4, 20 mmol/L sodium pyrophosphate, 3 mmol/L benzamidine hydrochloride, and 4 mmol/L glycerol 2-phosphate. After determining the protein concentrations using DC Protein assay dye (Bio-Rad), equal amounts of protein lysates (200-500 μg) were diluted in lysis buffer, and biotinylated proteins were incubated overnight with the streptavidin beads. The beads were subsequently washed 4-6 times the next day with lysis buffer. The biotinylated proteins are eluted by adding 50 μl of 2X Laemmli sample buffer and boiling the samples at 95°C for 5min. The samples were run on 10% gels and then electro-transferred to polyvinylidene fluoride membrane (PVDF, Millipore). The blots were probed with the appropriate primary and secondary antibodies. Enhanced chemiluminescent reagent (Santa Cruz Inc.) was used to visualize the labelled proteins.

In some experiments, the membrane and cytosolic fractions from hippocampal slices were separated by centrifugation at 13,000 g for 1 hour at 4°C and omitting the detergent (NP-40) from the solubilization buffer. The proteins from the membrane fraction were then resolved in normal solubilization containing NP40 (see protocol in Brust et al., 2007; Brust et al., 2006).

4.2.4 Drugs

The active Tat-GluA2-3Y peptide consists of the following amino acid sequence: YGRKKRRQRRRYKEGYNVYG, where Tat is YGRKKRRQRRR (the cell penetrating amino acid peptide sequence contained within the protein transduction domain of HIV gene called Tat), and YKEGYNVYG represents a GluA2 C-terminal amino acid sequence that interacts with the endocytic protein AP2 thus preventing GluA2 internalization (Ahmadian et al., 2004). The Tat-GluA2-3Y peptide and its scrambled version (scrambled Tat-GluA2-3Y: YGRKKRRQRRR-VYKYGGYNE) were purchased from GL Biochem Ltd. Forskolin and rolipram were obtained from Tocris. The dynamin inhibitor Dynasore was also purchased from Tocris. The drugs were dissolved in DMSO before being added to ACSF. The final concentration of DMSO was always < 0.01%.

4.2.5 Analysis

To normalize the protein bands from the membrane fractions, we used an anti-beta actin antibody (10,000; Sigma) or a monoclonal mouse anti-GAPDH antibody (1:2000; Millipore) to quantify the signal of GluA2/1 bands. Labelled protein bands were visualized using ECL. Densitometry analysis was performed using ImageJ software. Densitometry was performed using Quantity 1 (Bio-Rad) and ImageJ (public domain) computer programs. A single analysis of variance was performed to obtain the overall significance of the treatments followed by a post-hoc Student Newman-Keuls test to determine significance between specific treatments.

4.3 Results

4.3.1 Decreased associations of A1Rs and AMPARs in middle-aged rat hippocampus

Previously, we showed an association of adenosine receptors (specifically A1Rs) and AMPARs in hippocampus of young (<30 days old) rats. We next investigated whether a different level of association between A1Rs and AMPARs was displayed in the hippocampus of middle-aged rats. Using coimmunoprecipitation experiments, I showed that both GluA2 and GluA1 subunits were found in the A1R immunoprecipitates from young and middle-aged hippocampal homogenates. In Figure 4.3.1, the association of GluA2 with A1R was significantly decreased in middle-aged rats compared to that in young rats. Similarly, the level of co-precipitation of GluA1 and A1R was also lower in middle-aged rats compared to that in young rats. This decreased level of coimmunoprecipitation between A1Rs and AMPARs might reflect changes in the overall expression of either proteins, and this is addressed further below (see Figure 4.3.4). These results indicate age-dependent modification of the association between A1Rs and AMPARs.

4.3.2 Aging-related decrease in biotinylated surface AMPARs and A1Rs

Aging is associated with biochemical, anatomical and physiological changes in the central nervous system (Rosenzweig and Barnes 2003). Glutamate is the principal mediator of excitatory synaptic transmission in the hippocampus. Based on our recent report (see Chen et al., 2014), we hypothesized that activation of functional A1Rs expressed on cell surface would also induce a decrease in AMPAR surface expression levels in older hippocampal brain slices. To investigate this possibility, the present study assessed whether there were age-related changes in AMPAR expression in hippocampal membrane fractions in young versus middle-aged rats. When homogenized hippocampal slices were centrifuged to separate the membrane fractions, both GluA1 and GluA2 expression levels decreased in the membrane fractions of old rats as shown in Figure 4.3.2. Analysis of membrane fractions revealed significant differences in GluA1 and GluA2 immunoreactivities in older rat brains, starting at 3 months of age. Compared to 1 month-old rats, GluA1 immunoreactivity was 37.96 ±12.90 % in 3 month-old rats, 43.26

±14.74% in 6-7 month-old rats, and 19.32 ± 3.40% in 10-12 month-old rats. The levels of GluA2 in membrane fractions were similarly reduced to levels comparable to those seen in GluA1. In the hippocampus, GluA2 immunoreactivity was 41.83±1.89% in 3 month-old rats, 35.19±4.28% in 6-7 month-old rats, and 23.52±5.58% in 10-12 month-old rats. All of these values were compared against 1 month-old rats. These results suggest that expression levels of GluA1 and GluA2 in membrane fractions were reduced in older hippocampus, as early as at 3 months of age. The majority of these proteins from membrane fractions are presumably derived from plasma membranes, but a more direct assessment of changes in the localization patterns of proteins located in the plasma membrane is addressed below using biotinylation assays (see Figure 4.3.2B). Taken together, there is a significant aging-related decrease in membrane fractions of levels of AMPAR GluA1 and GluA2 subunits, which is consistent with the presence of increased adenosine tone in aged rats and the heteromeric association of GluA2 and GluA1 subunits.

To confirm whether age-related reduction exists in surface expression of hippocampal AMPARs as suggested from my membrane fractionation results, I then performed biotinylation experiments for more direct assessment of surface expression of AMPARs and adenosine receptors. Hippocampal brain slices of both one month and one year old rats were incubated with NHS-SS-Biotin, and then washed and lysed with detergent-containing (1% NP40) lysis buffer. Biotinylated proteins were isolated with streptavidin beads. Western blots were performed to evaluate the effects of aging on the relative protein levels of AMPARs in the hippocampus. Consistent with my membrane fractionation results, the biotinylation results also revealed a significant decrease in GluA1 and GluA2 AMPAR subunits in middle-aged rats (12 months old) compared to 1 month-old rats, as shown in Figure 4.3.2B. Furthermore, to determine whether surface levels of adenosine receptors also differed between young and middle-aged rats, biotinylated surface levels of A1R and A2AR were tested. The results showed a significant decrease in A1R but an increase in A2AR in the middle-aged rat hippocampus. Thus, the results of this experiment show an age-related decrease in biotinylated surface levels of A1R, GluA1 and GluA2, but increase in A2AR in middle-aged rats.

Figure 4.3.1

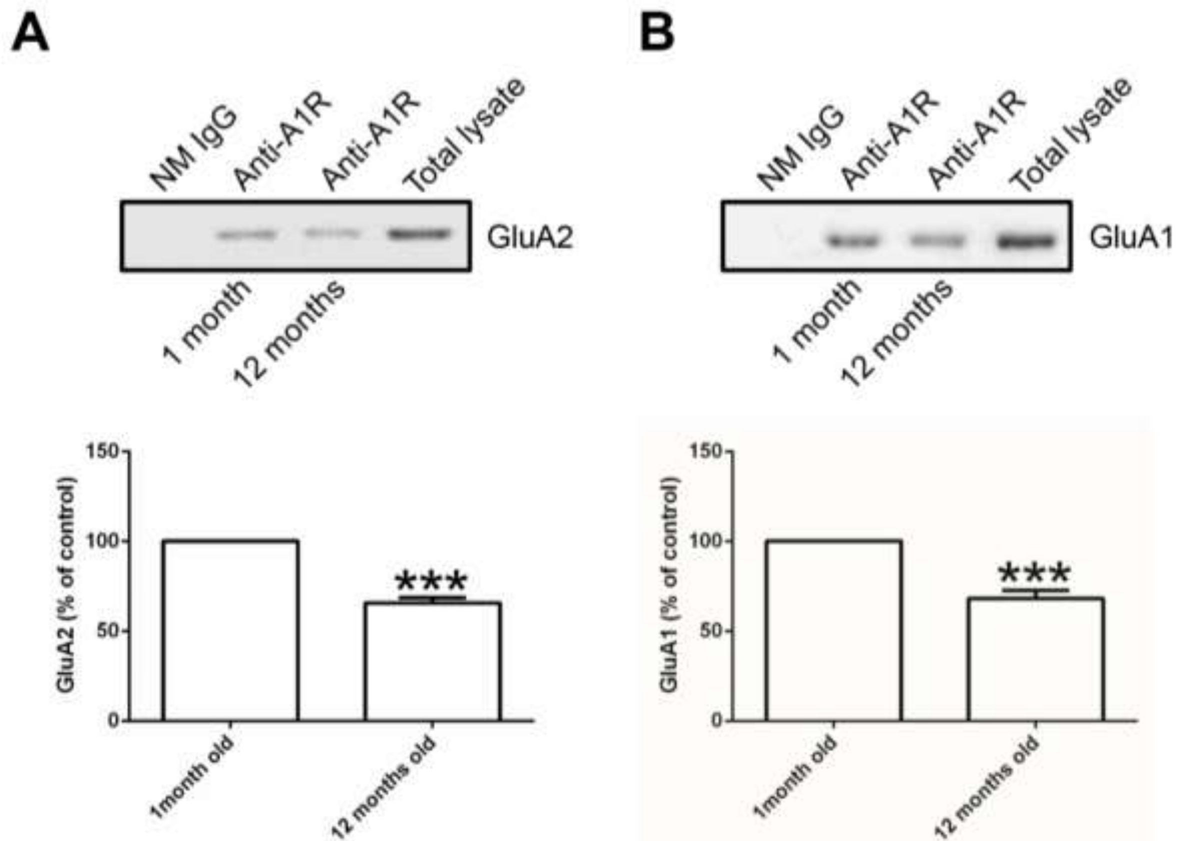


Figure 4.3.1 Immunoprecipitation results showed less interaction between A1Rs and AMPARs in 12 months old hippocampus compared to 1 month old hippocampus. A. Anti-A1R antibody was used to perform immunoprecipitation. I found less co-precipitation of GluA2 with A1R (A, lane 3) in 12 months old hippocampus compared to that in 1 month old hippocampus (A, lane 2). Total lysates were from 1 month old rat hippocampus (A, lane 4). B. In anti-A1R immunoprecipitated protein complex, I also found less GluA1 co-precipitating with A1R (B, lane 3) in 12 months old hippocampus compared to that found in 1 month old hippocampus B, lane 2). Total lysates were from 1 month old rat hippocampus (B, lane 4). Data represent means ±SEMs. Statistical significance comparing young and older brains was assessed by paired t-test (***P<0.001).

Figure 4.3.2

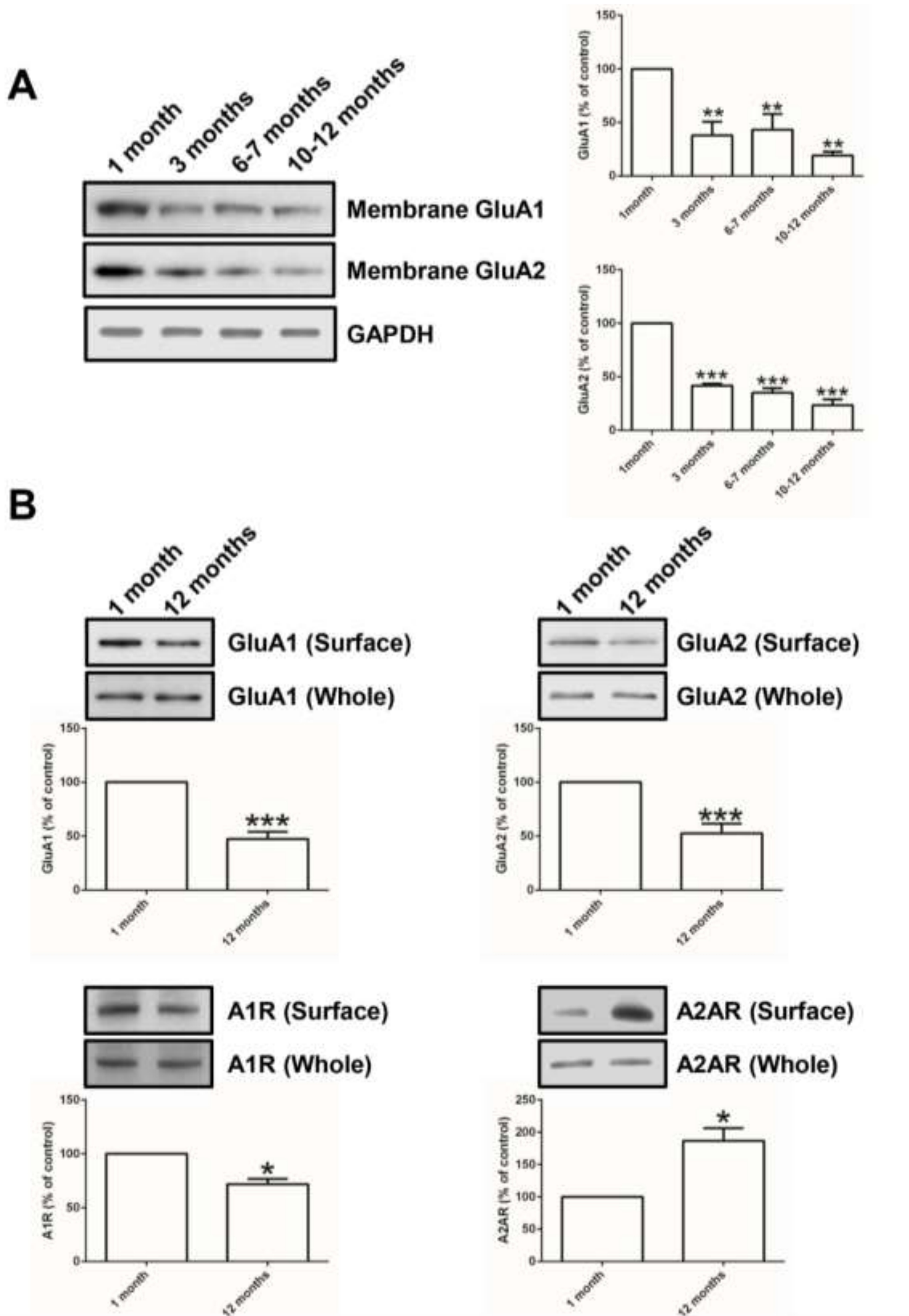


Figure 4.3.2 AMPAR levels decreased during aging in the plasma membrane of rat hippocampus. (A). Representative western blots show levels of GluA1 (106kDa), GluA2 (102 kDa) and GAPDH (37kDa) immunoreactivity in plasma membrane fraction from 1, 3, 6-7, and 10-12 months old rats. Graphs show the levels of GluA1, GluA2 and GAPDH immunoreactivity assessed by densitometry analysis of western blot ECL films. Membrane levels of GluA1 or GluA2 were normalized to the respective levels of GAPDH immunoreactivity. (B). Biotinylation results showed that biotinylated surface levels of GluA1, GluA2, and A1R decreased, while surface levels of A2AR increased. Values are means±SEMs. Statistical significance of values compared with control (1 month old) was assessed by one-way ANOVA. (**P<0.01, ***P<0.001).

4.3.3 Aging impaired cLTP induction and maintenance

Numerous early studies (Barnes, Rao et al. 1992; Bergado and Almaguer 2002; Dieguez Jr and Barea-Rodriguez 2004) have already suggested that aged brains of rodents show deficiencies in LTP, and previous studies also suggested that the cellular mechanism underlying the deficits in synaptic plasticity in old brains could involve adenosine signaling (Rex, Kramar et al. 2005). However, the precise cellular mechanisms in adenosine-induced synaptic impairment in aged brains are largely unidentified. To induce cLTP in rat hippocampus, forskolin and rolipram were applied for 10 min in Mg^{2+}-free ACSF solution both in young and old hippocampal slices. A comparison between young (1 month-old) and middle-aged (12 month-old) rat hippocampal slices revealed a significant difference in their fEPSP responses to cLTP in the CA1 region. Figure 4.3.3A shows trace recordings of averaged fEPSPs obtained from a young rat (left) and a middle-aged rat (right) before (1) and after (2) cLTP. cLTP produced a larger increase in the fEPSP slope in young compared to middle-aged rats at the end of the 10 min cLTP drug cocktail, suggesting that LTP induction is attenuated in middle-aged hippocampal slices. After the 10 min cLTP protocol, a one-hour fEPSP recording was performed to determine whether the maintenance of LTP was also affected in the middle-aged brains. As shown in Figure 4.3.3B-C, when fEPSPs were compared before (see region of fEPSP traces denoted by “1”) and after cLTP (see region of fEPSP traces denoted by “2”), the young rats showed a cLTP of 253.83±27.69% with n=16 whereas the middle-aged rats showed a cLTP of only 180.00 ± 15.86% with n=17. Taken together, these results suggest that aging impairs chemical LTP induction and maintenance.

Figure 4.3.3

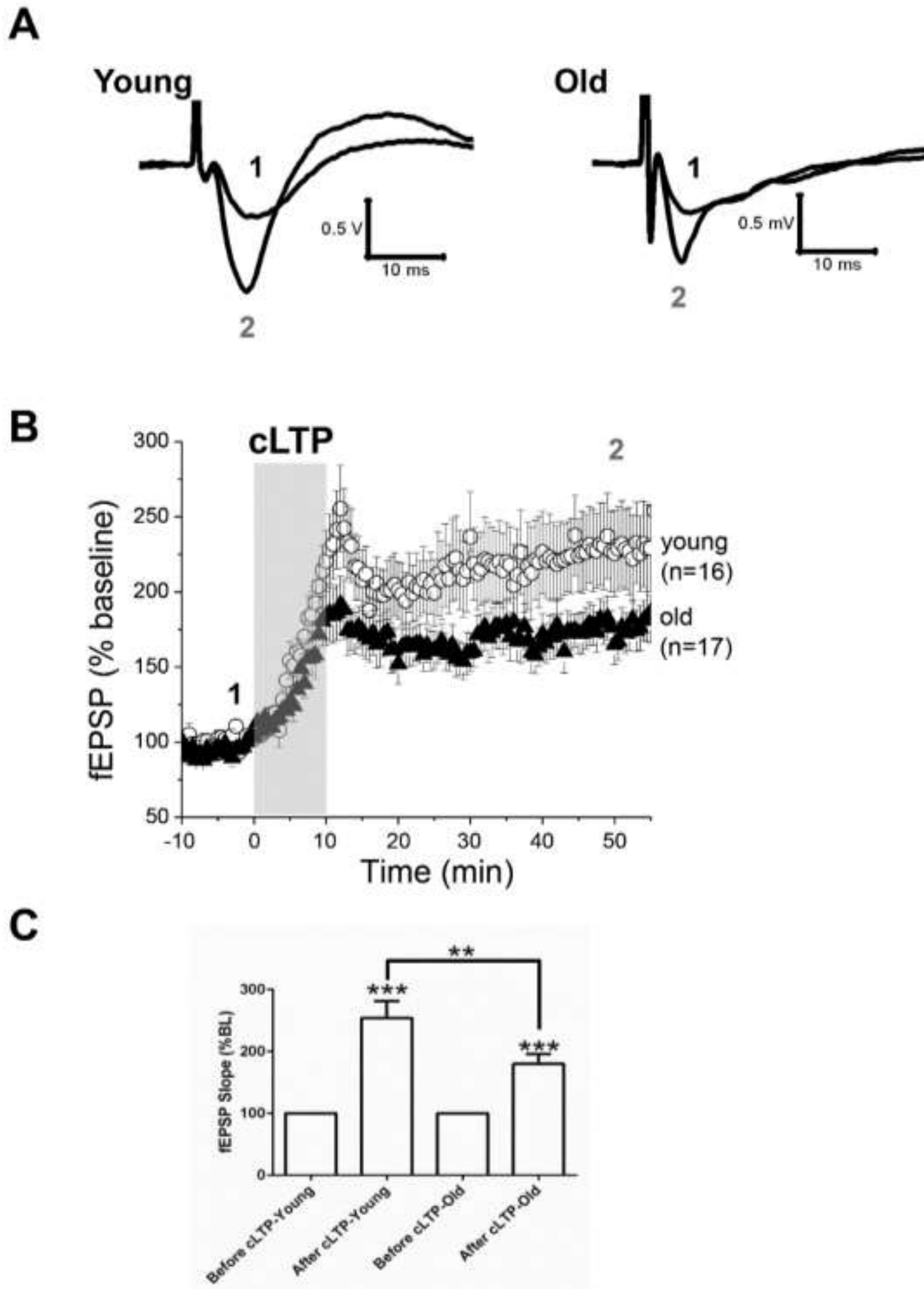


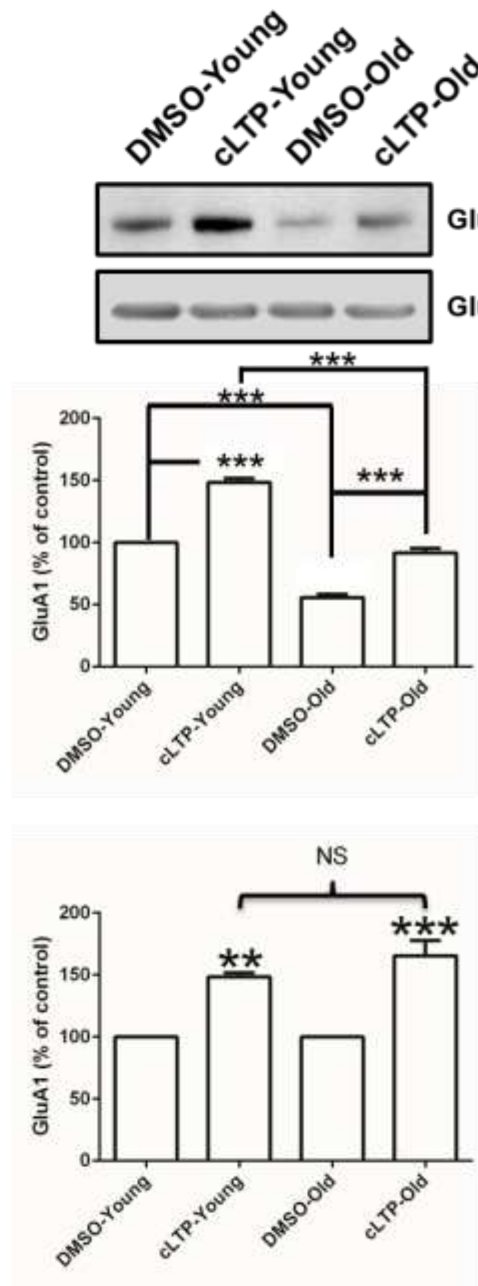
Figure 4.3.3 cLTP was impaired in old rats. A. The recordings of representative experiments are shown; each recording is the average of five consecutive responses obtained before cLTP and 60 min after cLTP induction in both young and old hippocampus. B. Averaged time course changes of fEPSP slope induced by cLTP in young (1 month old) and middle-aged (12 months) hippocampus. C. The effect of 10 min forskolin and rolipram on cLTP value in young and old hippocampus. All values are means±SEMs. Statistical significance of values compared with control (1 month old) was assessed by one-way ANOVA. (**$P<0.01$, ***$P<0.001$).

4.3.4 Effect of cLTP on surface levels of AMPARs in young and middle-aged rats

Next, I investigated whether biotinylated surface levels of AMPARs are altered after cLTP with forskolin/rolipram treatments in young and middle-aged rats. The results showed that the surface levels of GluA2 and GluA1 increased with forskolin/rolipram treatment in both young rats and middle-aged rats (Figure 4.3.4). However, the total levels (from whole lysates, which include both surface and cytosolic proteins) of both GluA2 and GluA1 subunits did not appear to be different, not only between young and middle-aged hippocampal lysates but also in absence or presence of the cLTP treatments. When the cLTP levels of GluA1 or GluA2 in both young and middle-aged brains were normalized to their respective DMSO controls, there was no difference in the relative levels of cLTP-induced potentiation of GluA2 or GluA1 subunits (Figure 4.3.4A-B, bottom summary bar charts). This latter result suggests that the insertion trafficking of AMPARs during and after cLTP may not be significantly altered in middle-aged hippocampus compared to that in young hippocampus. Therefore, I then tested the hypothesis (see next) that the increased adenosine tone observed in aged brains may produce greater internalization of AMPARs by enhancing the activities of the clathrin-mediated endocytic protein machinery.

Figure 4.3.4

A.



B.

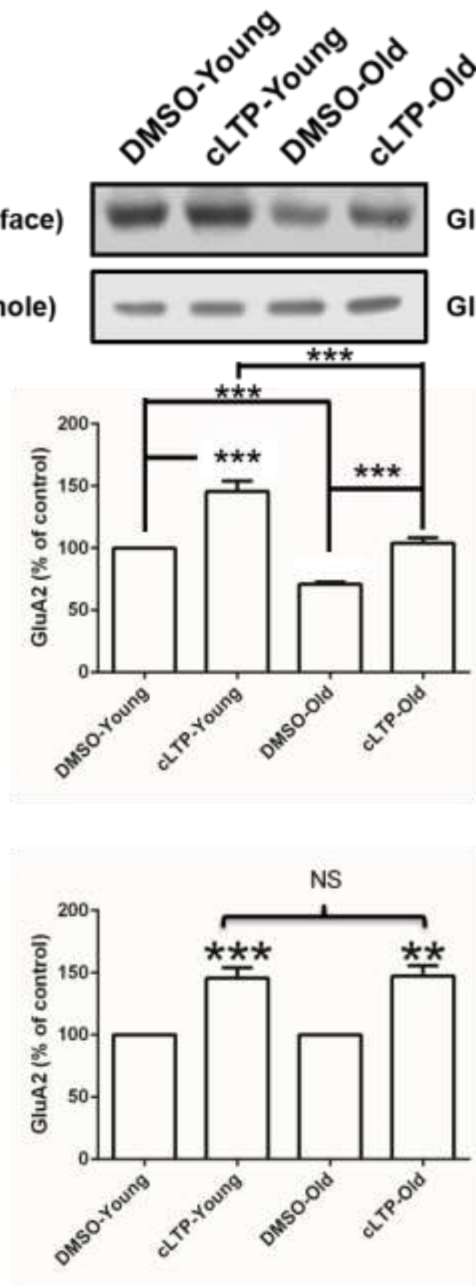


Figure 4.3.4 cLTP similarly increased AMPAR surface levels in hippocampal slices of old (12 months) and young (1 month) rats. A. 10 min treatment with forskolin and rolipram increased the biotinylated (surface) GluA1. Top. Biotinylated surface proteins from young and old hippocampus were precipitated by Streptavidin-agarose and immunoblotted by anti-GluA1 antibody. Bottom. Total lysate from young and old hippocampal slices were immunoblotted for GluA1. No changes were shown in the total (whole) GluA1 protein amount, while the surface GluA1 was more significantly increased after cLTP in young compared to old brains. B. Representative blots for GluA2 before and after cLTP. cLTP also increased surface expression of GluA2 in young brains and modestly increased surface GluA2 in old brains. Similar to GluA1, the total GluA2 expression level did not change in young and old hippocampal slices. The ratio of surface GluA1 or GluA2 to total GluA1 or GluA2 was graphed from three individual experiments. Data represent means±SEMs. Statistical significance of values compared with control (1 month old) was assessed by one-way ANOVA with post hoc test (**$P<0.01$, ***$P<0.001$). Summarized data were graphed from three individual experiments.

4.3.5 Endocytosis of AMPARs contributes to deficits in cLTP

We have recently shown that stimulation of A1Rs induced clathrin-mediated endocytosis of AMPARs (Chen et al., 2014, see also Chapter 2). Trafficking of AMPARs is crucial for LTP (Anggono and Huganir 2012), and AMPAR trafficking in postsynaptic membranes may be one of the most important mechanisms of LTP (Malinow and Malenka 2002). In addition, AMPARs have been previously shown to undergo clathrin-dependent endocytosis (Man, Lin et al. 2000), and the immediate early gene called *arg3.1/arc* (ARC) has also been shown to bind to the endocytic protein members endophilins 2 and 3 and dynamin 2 and causes activity-dependent internalization of AMPARs (Chowdhury, Shepherd et al. 2006; Nicoll, Tomita et al. 2006; Shepherd, Rumbaugh et al. 2006; Tzingounis and Nicoll 2006). Here, I tested the hypothesis that cLTP deficits may involve increased activity of endocytosis protein machinery. I examined the relationship between trafficking of AMPARs and cLTP levels in young and middle-aged brains. Specifically, I focused on determining whether blocking clathrin-mediated and dynamin-dependent endocytosis of AMPARs before cLTP could alter the biotinylated surface expression levels of AMPARs after treatments with forskolin and rolipram in young and middle-aged rat hippocampus. I pre-incubated hippocampal slices with Tat-GluA2-3Y peptide (2 μM), scrambled Tat-GluA2-3Y peptide (2 μM) or Dynasore (100 μM) before cLTP. The results show that AMPAR surface expression levels increased after 10 min of cLTP (Figure 4.3.5; see also Figure 4.3.4). Blocking GluA2 endocytosis with Tat-GluA2-3Y peptide dramatically enhanced the surface expression of both GluA2 and GluA1 subunits after cLTP in both young and middle-age brains. Similarly, blocking GluA2 endocytosis with Dynasore also enhanced GluA2 and GluA1 surface expression in both age groups. Conversely, pre-incubation of scrambled Tat-GluA2-3Y peptide did not alter the surface expression levels of GluA2 or GluA1 induced by cLTP. Together with the results above, these results suggest that clathrin-mediated endocytosis of AMPARs is enhanced in middle-aged brains, which likely contributes to the impairments in cLTP induction and maintenance in hippocampal brain slices.

Figure 4.3.5

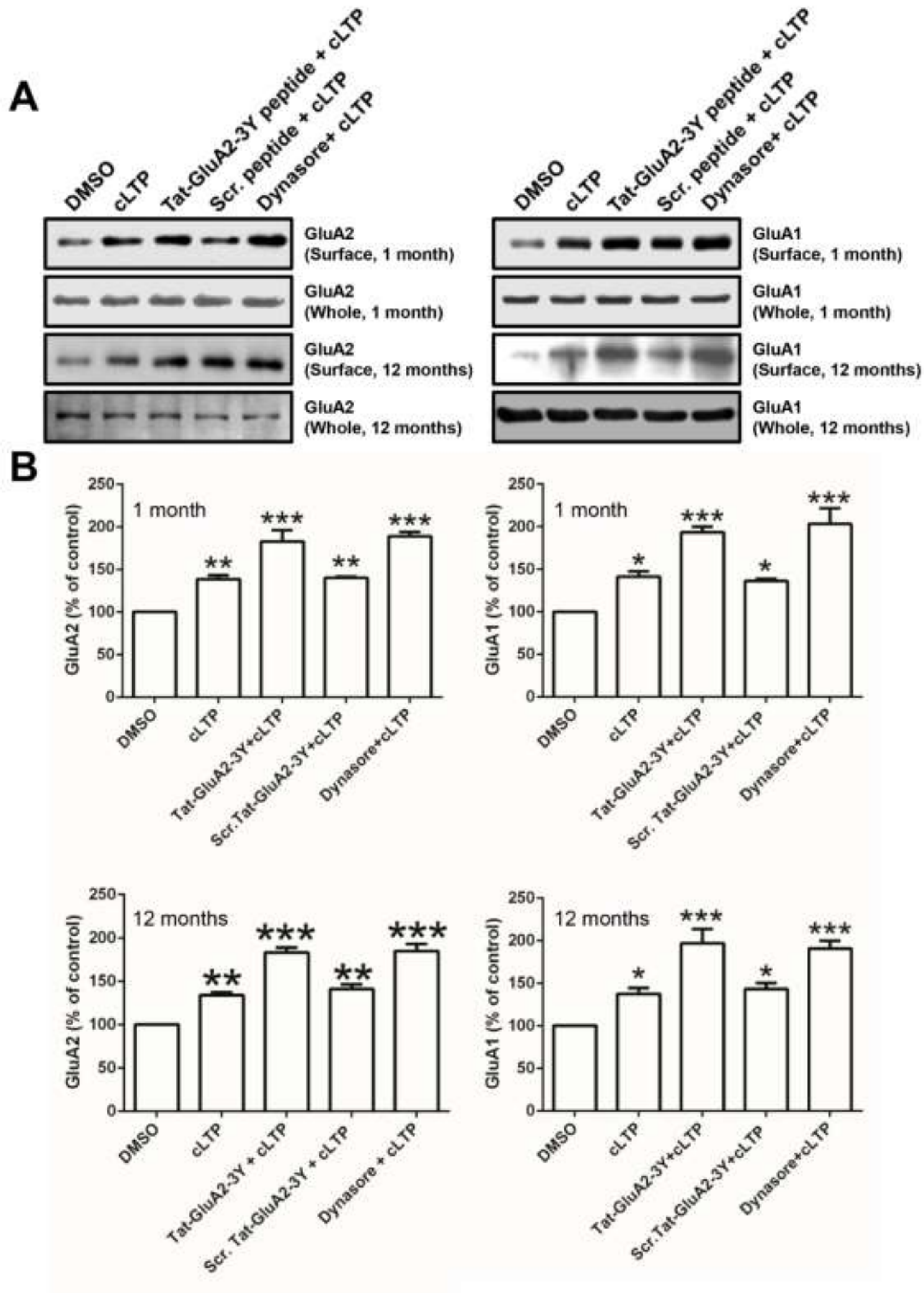


Figure 4.3.5 Dynasore and Tat-GluA2-3Y peptide enhanced the increase of GluA1 and GluA2 surface expression induced by cLTP in both young and old hippocampal slices. A. Top. Representative blots show biotinylated surface proteins and total proteins from hippocampal slices (young and old) treated with DMSO, forskolin and rolipram (cLTP), Tat-GluA2-3Y peptide + cLTP, scrambled Tat-GluA2-3Y peptide + cLTP, and Dynasore + cLTP. Blots were immunoblotted with anti-GluA2 or anti-GluA1 antibody. cLTP increased surface expression of GluA2 or GluA1 in both young and old hippocampal slices, but did not affect total GluA2 or GluA1 protein levels. These cLTP effects were further enhanced by one hour pre-incubation with Tat-GluA2-3Y peptide (2 μM) or Dynasore (100 μM) (to inhibit endocytosis of GluA2 and GluA1), but not with scrambled Tat-GluA2-3Y peptide. B. top. Summary chart of surface levels of GluA2 (B top, left panel) and GluA1 (B top, right panel) in 1 month rat hippocampus. B bottom, same as B top, GluA2 and GluA1 immunoreaction in middle-aged hippocampus with or without clathrin-mediated endocytosis are shown. Statistical significance of values compared with control (DMSO control in one month old hippocampal slices) was assessed by one-way ANOVA. (*P<0.05, **P<0.01, ***P<0.001).

4.4 Discussion

The main finding of the present study is that there is a decreased association of A1Rs and AMPARs in the hippocampus of middle-aged rats compared to young rats, this is accompanied by decreased cLTP in middle-aged rats, which is consistent with previous studies (Barnes and McNaughton 1985; Ward, Oler et al. 1999). However, we also reported that surface levels of A1Rs and AMPARs are significantly reduced, while the A2AR surface levels are dramatically enhanced, in the hippocampus of middle-aged rats compared to young rats. This suggests that changes in adenosine tone and adenosine receptors in older brains may alter the expression of AMPARs and induce adaptations in glutamatergic synaptic transmission. Previous evidence suggests that AMPAR trafficking is critical for synaptic plasticity (Man, Lin et al. 2000; Liu and Zukin 2007). Defects in trafficking of AMPARs cause deficiencies in synaptic plasticity during aging (Henley and Wilkinson 2013). Here, my results suggest that the trafficking and the overall surface expression of AMPARs before cLTP reflect different levels of adenosinergic signaling in the hippocampal slices of young and old animals. The observation that A1Rs are dramatically reduced while A2ARs are increased on the surface membranes of older brains may be a direct consequence of the increased adenosine tone which likely contributes to increased desensitization of A1Rs. We have recently observed a similar reduction of A1Rs after A1R stimulation with agonist CPA or after 48 hours of the pial vessel disruption (PVD) focal cortical stroke injury model (Chen, Xiong et al. 2014). We have also observed an increase in A2AR surface expression in this PVD model. Currently, the cellular mechanisms for this A2AR upregulation in the PVD model as well as in middle-aged brains (present study) are undefined. Interestingly, the levels of the protein kinase CK2 that are subsequently presented in Chapter 5 appears to be reduced in cerebral ischemia, and this pattern is also documented in aging brains (see review by (Blanquet 2000)). Since a previous study (Rebholz, Nishi et al. 2009) also reported that CK2 is critical for A2AR desensitization, it is possible that the increased A2AR surface expression in middle-aged hippocampus reflects a reduction in the levels or activities of CK2. Therefore, an increase in A2AR signaling and a decrease in A1R signaling could regulate AMPAR-mediated glutamatergic synaptic plasticity in older brains.

Furthermore, I observed that cLTP increased the surface expression of AMPARs to similar levels in the hippocampus of young and middle-aged rats, suggesting that surface insertion trafficking pathways are not likely different between younger and older brains. However, further studies are needed to confirm whether the phosphorylation of AMPARs (at Ser845 and Ser831 for GluR1 or at Ser880 for GluA2) is an important mechanism involved in AMPAR surface insertion during aging.

In contrast, I found that the fEPSPs were significantly lower after cLTP treatments of middle-aged hippocampal slices compared to young ones, which might suggest that the endogenous levels of activity of clathrin-mediated protein machineries may be elevated in older brains; however, this possibility also requires further investigations. I also found that pretreatments of hippocampal slices with endocytosis inhibitors in young and old brains caused further enhancements in the surface expression of GluA2 and GluA1, which suggests that inhibiting the endocytic pathways may be sufficient to minimize the effects of endogenous adenosine tone in both young and older brains. Moreover, these results also suggest that aging decreased the surface levels of AMPARs which leads to impaired synaptic plasticity.

It is well known that extracellular concentrations of adenosine increase in aging rats (Sperlagh, Zsilla et al. 1997). As our results indicate (Chen, Xiong et al. 2014) (also see Chapter 2), activation of A1Rs decreases surface expression of AMPARs. Therefore, we predicted that more functional A1Rs in older hippocampus would be detected and induce a reduction in surface AMPAR expression. As demonstrated in figure 4.3.2, AMPAR levels decreased in both membrane fractions and biotinylated surface expression, which provided support to our hypothesis that increased adenosinergic signaling would downregulate AMPARs. However, the A1Rs are also downregulated in aging brains, suggesting that increased adenosinergic signaling is expected to affect not only glutamatergic synaptic transmission but also adenosine receptor trafficking.

Similar results have been found by other groups in terms of age-related decreases in AMPAR expression in aging brains (Sonntag, Bennett et al. 2000). Impairments in LTP occur during aging and this impairment is likely attributable to defects in AMPAR trafficking (Henley and Wilkinson). Other reports have also demonstrated a reduction in the expression and density of A1 in cortical and hippocampal regions in older rat brains

(Pagonopoulou and Angelatou 1992; Cunha, Constantino et al. 1995; Cheng, Liu et al. 2000). Previously, we showed there was a physical interaction between A1Rs and AMPARs (Chen, Xiong et al. 2014). Here, we showed a decrease in the surface expression of A1Rs and AMPARs in older hippocampus, which may indicate that the decreased protein complex of A1Rs and AMPARs at the surface of hippocampal neuronal membranes of older hippocampus may explain the deficits in cLTP.

In senescence-accelerated prone mouse strains and resistant strains, age-related loss of A1Rs and age-related increase in A2ARs were detected (Castillo, Albasanz et al. 2009). Modulation of postsynaptic AMPARs in the brain by phosphorylation may play a role in the expression of synaptic plasticity at central excitatory synapses. cLTP stimulation resulted in significant increases in phospho-Ser-845, suggesting there was an increase in surface GluA1 with cLTP stimulation regardless of basal stimulation (Oh, Derkach et al. 2006). Antagonists of A2AR prevented aging dependent memory deficits (Prediger, Batista et al. 2005; Costa, Botton et al. 2008). It was reported that the density of A1R-DPCPX binding sites was downregulated in aged rats but CGS 21680 binding ability of A2AR increased (Cunha, Constantino et al. 1995; Canas, Porciuncula et al. 2009), which are consistent with our biochemical results.

Although adenosine signaling has been studied for decades, much more work is required to develop a more complete understanding of this phenomenon. This study extends our understanding of the interaction between A1Rs and AMPARs and the functional consequences of this interaction on the cellular distribution of AMPARs in aged rats. The findings from this study add further insight into the molecular and cellular mechanisms of AMPAR trafficking that underlie the defects in synaptic plasticity during aging. Further studies are needed to elucidate the importance of activity-dependent alterations in the surface expression of AMPARs and adenosine receptors that accompany impairments in synaptic plasticity that are associated with aging-related neurological disorders.

CHAPTER 5

General Discussion

5.1 A summary of the main findings

The goal of this dissertation was to elucidate novel mechanisms of adenosinergic signaling pathways, which could be exploited for developing neuroprotective therapies in stroke. More specifically, I found a novel interaction between AMPARs and adenosine receptors, as well as interaction between adenosine receptors and nucleoside transporters called ENTs, and characterized these interactions using different but complementary techniques, including expression levels in membrane fractions, biotinylated surface levels of receptors, coimmunoprecipitation and co-localization of receptors, phosphorylation level of receptors, and surface immune-staining density of receptors on cultured hippocampal neurons. I have also demonstrated the regulation of adenosine receptors and nucleoside transporters by CK2. Using an animal model of small vessel stroke (i.e., the focal cortical ischemia evoked by pial vessel disruption) and comparing these to sham animals, I have also examined the alterations in the surface levels of AMPARs, adenosine receptors and ENTs in hippocampus of pial vessel disruption cortical stroke model. Finally, I explored the consequences of increased adenosinegic signaling by further investigating the interactions between AMPARs and adenosine receptors during aging. My results are summarized in the schematic diagram shown in Figure 5.1.

The connection between Chapter 2 and Chapter 3 is that adenosine receptors and transporters are both important for adenosine signaling and for regulating levels of neurodegeneration. In Chapter 2, I found a novel interaction between adenosine receptors (in particular, A1Rs) and AMPARs, which leads to activation of intracellular protein kinases (e.g., p38 MAPK and JNK) and phosphatases (e.g., PP2A) which contribute to a long-lasting synaptic inhibition we have called adenosine-induced persistent synaptic depression (or APSD). My data obtained from an *in vivo* focal cortical ischemia model also supports the model that these intracellular signaling

Figure 5.1

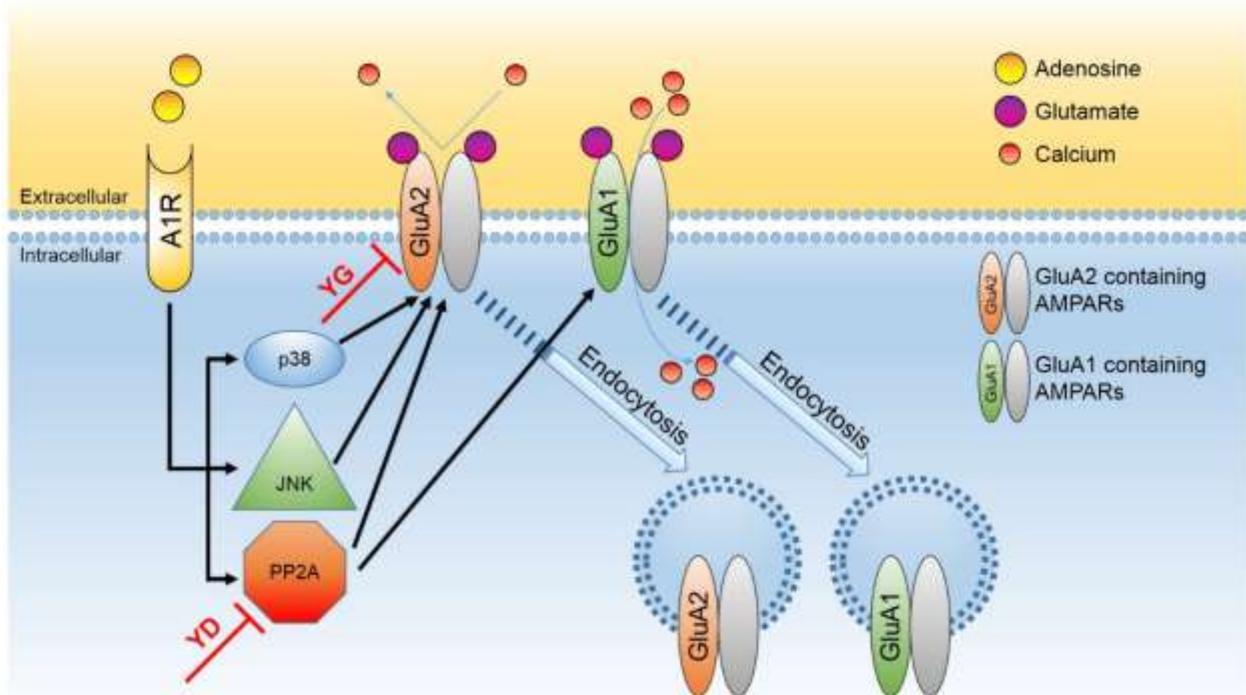


Figure 5.1 Schematic representation of the molecular mechanism of prolonged A1R induced AMPAR endocytosis. Stimulation of A1R induced endocytosis of GluA2 containing AMPARs through p38 MAPK, JNK and PP2A. However, activation of A1R-induced GluA1 AMPAR endocytosis was mediated by PP2A activation, but not by p38 MAPK and JNK activation. GluA2-containing AMPARs are calcium impermeable. To prevent the A1R-induced AMPAR endocytosis, both YG peptide (Tat GluA2-3Y peptide, YGRKKRRQRRRYKEGYNVYG), which inhibits GluA2-containing AMPAR endocytosis, and YD peptide (amino acid sequence of YD is not described here due to patent application in progress), which prevents PP2A activation, prevented A1R-induced endocytosis of AMPARs.

pathways contribute to the clathrin-mediated internalization of GluA2-containing AMPARs, which suggests that loss of these GluA2 AMPAR subunits ultimately leads to increased neurodegeneration. However, more studies are required to establish a direct link between increased adenosine signaling and stroke-induced neurodegeneration. For example, the contributions of both presynaptic and postsynaptic mechanisms to adenosine-induced synaptic depression and neurodegeneration need further investigation using more direct electrophysiological analyses, for example, with direct recordings of excitatory postsynaptic currents (EPSCs) from postsynaptic membranes or from direct measurements of miniature excitatory postsynaptic currents (mEPSCs) to determine directly whether APSDs are a direct consequence of a presynaptic or postsynaptic mechanism or both. One of the reasons we studied adenosine signaling is that extracellular adenosine levels are enhanced during hypoxia and stroke (Van Wylen, Park et al. 1986; Phillis, Walter et al. 1987; Dale, Pearson et al. 2000). Adenosine transporters are important in controlling extracellular adenosine levels during hypoxia and stroke (Chakrabarti and Freedman 2008; Soylu, Zhang et al. 2012; Cui, Bai et al. 2013). However, the regulation of adenosine transporters is not well studied. Since ENTs contain consensus sites for casein kinase 2 (CK2) phosphorylation (canonical sequence S/TXXD/E) (Stolk, Cooper et al. 2005), we hypothesized that CK2 regulates the phosphorylation level of ENTs. In Chapter 3, we have shown that the inhibitors of CK2 decreased the phosphorylation of ENT1 at Serine 254, a well-known consensus site for CK2 phosphorylation (Bone, Robillard et al. 2007). The inhibition of CK2 also decreased the surface levels of ENTs. Since both surface and phosphorylated levels of ENTs were decreased in the presence of CK2 inhibitors, this suggested that the overall levels of functional ENTs were attenuated, which would be expected to increase adenosine tone. Indeed, we found using electrophysiological analysis that adenosine tone increased in the presence of CK2 inhibitors (unpublished observations). Whether this apparent rise in adenosine tone (unpublished data from Chen, Stockwell and Cayabyab) from prior inhibition of ENT surface expression would produce neuroprotection, has never been previously reported. A previous report (Zhang, Xiong et al. 2011; Soylu, Zhang et al. 2012), however, showed that overexpression of ENT1 led to a decreased protective function and instead produced increased infarct volumes in an endothelin model of stroke injury.

Consistent with this previous report from Parkinson and colleagues, we demonstrated that preincubation of rat hippocampal slices with CK2 inhibitors decreased ENT1 and ENT2 surface levels which was accompanied by decreased neuronal death in our hypoxic/reperfusion injury model (Chapter 3). However, whether decreased ENT1 or ENT2 surface expression is truly neuroprotective is not clearly established by our studies. There are ENT1 knockout mice that are available, which could be used to further elucidate the role of ENT1 in neuroprotection (Choi, Cascini et al. 2004). Nevertheless, our *in vitro* data from hypoxic/reperfusion injury model did indicate, however, that the reduction in ENT1 and ENT2 surface expression in the presence of CK2 inhibitors was accompanied by decreased neuronal cell death (see Chapter 3 and Figure 5.2). Whether an experimental intervention that limits the function of ENTs in animal models of stroke, such as the pial vessel disruption used in my studies, can be demonstrated to produce neuroprotection as we have shown in our *in vitro* experiments (Chapter 3), remains to be determined. It is difficult to confirm our *in vitro* results using the CK2 pharmacological inhibitors with *in vivo* animal stroke models, since those reagents may have limited blood-brain barrier permeability. Future studies using CK2 knockout animals or Tat-peptide interference strategies are therefore required to further define the role of CK2 inhibition *in vivo* in downregulating ENT surface expression, and whether this represents a possible neuroprotective target in animal stroke models.

The connection between Chapters 2 and 4 is that the extracellular adenosine levels appear to be increased in pathological conditions such as stroke, as well as during aging. We observed that the activation of adenosine A1Rs was able to induce AMPAR endocytosis under both ischemic (PVD model) and hypoxic conditions. We wanted to know whether A1Rs can induce AMPAR endocytosis during aging. Therefore, we compared the surface levels of AMPARs and also determined whether deficits in LTP levels in aged brains (12 months) compared to young brains (one month) can be correlated with increased adenosinergic signaling which mediates increased clathrin-mediated AMPAR endocytosis. We found that the levels of both AMPARs and LTP were lower in aged rats compared to those of young rats. AMPAR endocytosis in both young and aged rats occurred through clathrin-mediated endocytosis pathway, and this endocytic pathway appeared to be intact in both younger and older rat brains. I also found

that AMPAR interaction with A1Rs was preserved in aged rat brains, as was shown by my coimmunoprecipitation studies. However, it will be important to determine in future studies whether excessive adenosine level was absolutely critical to the downregulation of AMPAR surface expression, which leads to impairments in LTP induction and maintenance. It is also essential to identify the adenosine-related proteins that could contribute to this elevation in adenosine tone in aged brains. For example, some possible candidates could include decreased adenosine kinase levels or activities, increased ecto-5'-nucleotidase (or CD73) levels, increased ecto-phosphodiesterases, and decreased ENT levels or activities, to name a few (see Figure 5.4). Future studies are also required whether components of clathrin-mediated endocytosis pathway, including dynamin, endophilin, and protein phosphatase, are upregulated during aging to mediate some of the LTP attenuation. Taken together, my results from Chapters 2 and 4 indicate that adenosine induced AMPAR endocytosis is a novel mechanism for attenuating LTP in aged rats. This is significant since cognitive decline is often reported in aging-related neurological diseases, such as Alzheimer's disease, Parkinson's disease, and stroke (Wilson, Leurgans et al. 2011; Lindenberger 2014).

As shown in Figure 5.1, stimulation of A1R activates the p38 MAPK and JNK, and results in translocation of PP2A to plasma membrane fractions (Brust, Cayabyab et al. 2006; Brust, Cayabyab et al. 2007). Pharmacological inhibitors of p38 MAPK, JNK and PP2A all prevented A1R-induced endocytosis of GluA2-containing AMPARs. Numerous mechanisms are known to be involved in the decreased levels of surface-expressed AMPARs, specifically those containing the GluA2 subunits. These mechanisms may include the involvement of the immediate early gene called *arg3.1/arc* (ARC) (Chowdhury, Shepherd et al. 2006; Shepherd, Rumbaugh et al. 2006; Tzingounis and Nicoll 2006) which has been shown to associate with the endocytic proteins endophilins 2 and 3 and dynamin 2 to regulate AMPAR trafficking. Since ARC is transcribed and targeted to dendrites during periods of information processing and storage (Chowdhury, Shepherd et al. 2006), similar to periods during LTP or LTD induction, and our current results showed that prolonged A1R stimulation leads to clathrin-mediated internalization of GluA2-containing AMPARs, together these previous results and our current findings point to a possible involvement of ARC in A1R-mediated endocytosis of AMPARs. However,

more work is needed to show a link between AMPAR downregulation and a possible increase in ARC levels during prolonged A1R stimulation or during *in vivo* focal cortical ischemia. Another mechanism that may contribute to decreased GluA2 surface expression is increased Ser880 phosphorylation (Chung, Xia et al. 2000; Seidenman, Steinberg et al. 2003; Hu, Huang et al. 2007). Previous studies (Thomas, Lin et al. 2008; Ahn and Choe 2010) have also reported that increased JNK activation leads to increased Ser880 phosphorylation, which subsequently produced increased internalization of GluA2 subunits. It has been suggested that inhibition of JNK (by inhibiting or preventing the binding of JNK-interacting protein-1 to JNK) should lead to decreased Ser880-GluA2 phosphorylation, which should produce decreased GluA2 internalization. Whether Ser880-GluA2 is hyper-phosphorylated after prolonged A1R stimulation remains to be determined, but could potentially provide an alternative mechanism to explain A1R mediated GluA2 receptor endocytosis. Yet another potential mechanism that could be involved in downregulating GluA2 surface expression after excessive A1R stimulation is the possible decrease in interaction between NSF and GluA2 subunits. This mechanism, if shown to be involved in A1R-mediated AMPAR downregulation, may suggest that excessive A1R signaling causes a decrease in the stability of AMPARs on neuronal membranes. However, further studies are needed to test this hypothesis. Also to continue to investigate possible mechanisms of AMPAR endocytosis, we have designed a YD peptide, which prevents PP2A activation, to inhibit A1R induced AMPAR endocytosis (see Figure 5.1, unpublished data). We have already demonstrated using another peptide, i.e., the Tat-GluA2-3Y peptide, the usefulness of Tat-peptide interference strategy in preventing GluA2 AMPAR endocytosis (Chapter 2), so therefore the Tat-YD peptide shows great potential in preventing A1R-mediated synaptic depression, clathrin-mediated GluA2 AMPAR endocytosis, and neurodegeneration. Our preliminary data showed both peptides could prevent hypoxia/reperfusion-induced neuronal cell death in hippocampal brain slices (Zhi, Chen, Stockwell, and Cayabyab, unpublished), as shown by propidium iodide staining previously shown in rat hippocampus (Noraberg, Kristensen et al. 1999; Pugliese, Traini et al. 2009), indicating that these two peptides are neuroprotective, at least under *in vitro* conditions. Future studies will also be needed to confirm whether these

peptides have neuroprotective capabilities in our *in vivo* focal cortical stroke model using our PVD stroke model.

In Chapter 2, I found that A1Rs and GluA2-containing AMPARs formed stable protein complexes in both the hippocampus and cultured hippocampal neurons. In contrast, adenosine A2ARs did not co-precipitate or colocalize with GluA2-containing AMPARs. In addition, my findings also revealed that not only A1Rs and AMPARs are both physically coupled but they are also functionally associated. Prolonged stimulation of A1Rs with the agonist CPA can cause adenosine-induced persistent synaptic depression (termed APSD) in hippocampal brain slices, and APSD levels were blunted by inhibiting clathrin-mediated endocytosis of GluA2 with the Tat-GluA2-3Y peptide. Moreover, prolonged CPA incubation showed a significant depletion of GluA2 and GluA1 surface expression from hippocampal brain slices and cultured hippocampal neurons, indicating adenosine receptors and AMPARs functionally associate. Tat-GluA2-3Y peptide or dynamin inhibitor Dynasore prevented the internalization of CPA-induced GluA2 and GluA1 internalization, suggesting internalization of AMPARs is through clathrin-mediated endocytosis pathway. Furthermore, a confocal imaging analysis confirmed that functional A1Rs rather than A2ARs are required for clathrin-mediated endocytosis of AMPARs in cultured hippocampal neurons. It was also reported that pharmacological inhibitors or shRNA knockdown of p38 MAPK and JNK prevented the A1R-mediated internalization of GluA2 but not GluA1 subunits. This suggests both p38 MAPK and JNK are involved in the activation of A1R-induced internalization of GluA2, but neither is involved in the internalization of GluA1. Tat-GluA2-3Y peptide or A1R antagonist DPCPX also prevented the hypoxia-mediated internalization of GluA2 and GluA1. Finally, in the pial vessel disruption focal cortical stroke model, reduced hippocampal GluA2, GluA1, and A1R surface expression and synaptic depression were seen in hippocampal slices from unilateral cortical lesioned brains compared to sham brains. These *in vivo* results are in agreement with our *in vitro* findings that excessive adenosinergic signaling results in AMPAR downregulation and decreased probability of transmitter release. Together, our results indicate a previously unknown mechanism for A1R-induced persistent synaptic depression involving clathrin-mediated GluA2 and GluA1 internalization in hypoxia/cerebral ischemia.

So far, we only explored the influence of adenosine signaling on AMPARs, but the specific roles of persistent adenosine A1R signaling in regulating NMDAR during and after ischemia have not been investigated previously by others. It was reported that NMDAR subtypes play different roles from AMPARs in ischemic neuronal death. For example, the inhibition of GluN2A-containing NMDARs triggered neuron death (Chen, Lu et al. 2008). In contrast, the inhibition of the GluN2B-containing NMDAR was neuroprotective after transient global ischemia in animal models (Chen, Lu et al. 2008). The roles of NMDARs during stroke were recently reviewed (Lai, Zhang et al. 2014). Moreover, NMDARs can be activated by adenosine receptors, in particular by A2ARs, which suggests that adenosine receptor-induced internalization of AMPARs may result indirectly from the modulation of NMDA receptor activity (Rebola, Lujan et al. 2008). Since our electrophysiology recordings were performed in the absence of NMDAR blockers, we could not rule out the possibility of NMDARs contributing to A1R-mediated APSDs. However, in our confocal analysis, we showed in hippocampal neuron cultures that prolonged CPA incubation with presence of TTX and NMDAR antagonist D-APV, still induced AMPAR endocytosis. The physical interaction between A1Rs and GluN2A/GluN2B was undetectable by our coimmunoprecipitation studies (Chen and Cayabyab, unpublished observations). However, more work needs to be done to confirm this result in future, and particularly whether a functional interaction between A1Rs and NMDARs contributes to AMPAR trafficking.

In order to understand the pathophysiology of stroke and to explore potential treatments, several animal stroke models have been developed (Xiong, Mahmood et al. 2013). In this study, we applied a modified model of pial vessel class II disruption (PVD model). Instead of the middle cerebral artery occlusion (MCAO), we used this modified model in our study. The reasons can be discussed from different aspects. In the MCAO animal stroke model (Nagasawa and Kogure 1989), the lesion severity can be manipulated by relying on the duration of the occlusion. Necrosis and apoptosis were both found in this model. Necrosis predominantly occurs in the infarct core in the cortex or striatum while apoptosis mainly happens in the penumbra, the area surrounding the infarct (Lipton 1999). In the penumbra, both neuroinflammatory and neurodegenerative processes can take place (Carmichael 2005). However, the method of suture insertion

carries a high risk of subarachnoid hemorrhage (Murphy and Corbett 2009). In addition, it involves invasive surgical procedures and the reproducibility is low (Murphy and Corbett 2009). Therefore, MCAO is not an ideal model for my study. In the human brain, most strokes occur in large arteries (>0.1 mm diameter), while one third of human strokes are the results of small-vessel ischemia (Greenberg 2006; Kitamura, Nakagawa et al. 2006). It is generally acknowledged that small-vessel strokes lead to lacunar infarctions. To mimic small volume stroke in the rat brain, another local stroke model has been developed – the modified pial class II vessel disruption (PVD) model which induces small-vessel stroke by disrupting class II vessels. This prevents hemorrhage and causes a cone-shaped lesion exclusively located in the cerebral cortex. This method also prevents larger variations in lesion sizes. In addition, this model is highly reproducible with a high survival rate (Hua and Walz 2006; Hua and Walz 2006; Cayabyab, Gowribai et al. 2013). Hence, we chose PVD stroke model for our study.

In Chapter 3, I have shown that both equilibrative nucleoside transporters and A1Rs are widely expressed in the hippocampus, and they regulate the extracellular adenosine level and induce synaptic depression during and after a cerebral ischemia, respectively. Since ENTs contain consensus sites for CK2 phosphorylation, I tested several CK2 inhibitors (TBB, DMAT, and DRB) to determine whether these inhibitors could reduce pSer254-ENT1 levels in whole lysates. My results confirmed that CK2 blockers not only decreased pSer254-ENT1 levels but also decreased both A1Rs and ENTs. To confirm these results further, I showed that the CK2 activator spermine when applied to acute brain slices did increase the surface expression of pSer254-ENT1 and A1Rs in biotinylation assays of hippocampal brain slices. Moreover, biotinylation of cultured hippocampal neurons revealed that both ENT1 and ENT2 surface expression were downregulated by CK2 inhibitors or direct ENT inhibitors, as well as by A1R agonist CPA, but not in the presence of A1R antagonist DPCPX.

Figure 5.2

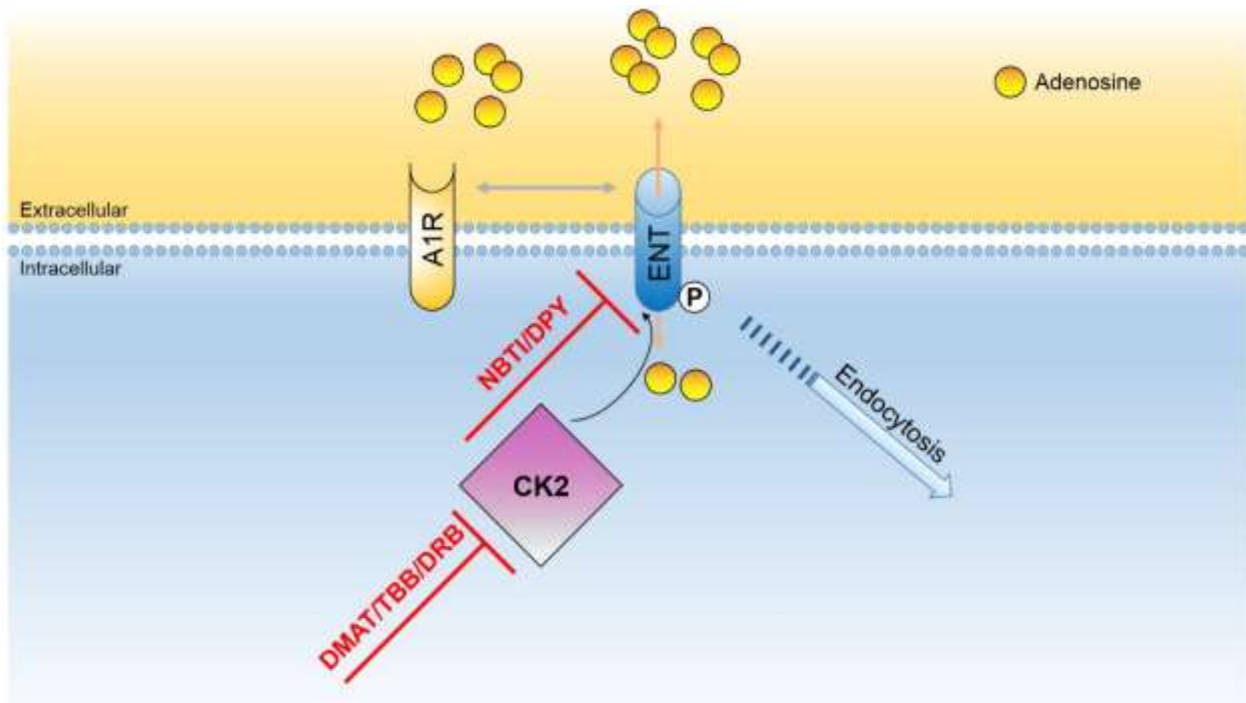


Figure 5.2 Inhibition of CK2 and ENT causes endocytosis of ENTs in rat hippocampus. Application of pharmacologicall inhibitors of CK2, such as DMAT, TBB or DRB, leads to endocytosis of ENTs. CK2 inhibitors also decreased the phosphorylation of ENT1 at Serine 254. ENT inhibitors DPY or NBTI results in endocytosis of ENTs but not attenuate the phosphorylation of ENT1. This reduction in ENT1 surface expression in the presence of either CK2 or ENT inhibitors was blocked by A1R antagonist DPCPX. In addition, a novel physical interaction between A1Rs and ENTs was observed by coimmunoprecipitation and colocalization in rat hippocampal slices and cultured hippocamal neurons (Chapter 3).

These results suggest that A1R and ENT1 are not only biochemically coupled but are also functionally associated. Although I have not yet demonstrated whether ENTs and A1Rs are co-internalized and trafficked to the same protein degradation pathways or to similar recycling vesicular pools, the similar patterns of decreased surface expression of ENTs and A1Rs after selective A1R stimulation and the colocalization of these proteins at or near the plasma membranes and intracellular membranes would suggest that some ENTs and A1Rs do traffic to the same subcellular localization after A1R signaling. Moreover, pretreatments of hippocampal slices with CK2 or ENT blockers also enhanced hypoxia-mediated downregulation of both ENT and A1R surface expression, further indicating that both proteins undergo similar trafficking in the presence of enhanced adenosinergic signaling in hypoxia. Therefore, these results strengthen our model that CK2-induced and A1R-linked ENT trafficking represents an important regulatory mechanism of hypoxic/ischemic hippocampal brain damage. My results showing that increased CK2 activity (with spermine treatments) leads to increased surface expression of A1Rs and ENTs, which accompanied the increased neuronal death after hypoxia/reperfusion injury, supports the role for increased CK2 activity in neurodegeneration. This is in sharp contrast to other previous reports (Hu and Wieloch 1993; Blanquet 2000; Kim, Jung et al. 2009) suggesting that CK2 levels and activity promote neuroprotection in cerebral ischemia. However, my own unpublished results suggest that CK2 levels are decreased in our PVD stroke model, and this decrease in CK2 levels was accompanied by decreased A1R and ENT surface expression (Chapter 3). It is clear that future studies are required to further clarify the roles of CK2 in neurodegeneration occurring in the different time periods following an ischemic damage. One possibility is that during the early phase (i.e., within minutes) of ischemic stroke, the CK2 activity is further increased which leads to increased phosphorylation and increased surface trafficking of the calcium-permeable GluA1 AMPAR subunits, as has been shown in cultured neurons (Lussier, Gu et al. 2014). Whether this CK2 regulation of GluA1 subunits contributes to the initial cascades of events that lead to increased neurodegeneration, remains to be determined. In contrast, the decreased levels of CK2 that I have observed two days after PVD stroke injury (Figure 5.3) was accompanied by decreased GluA1 (and GluA2) AMPAR subunits (Chapter 2). However, further studies

Figure 5.3

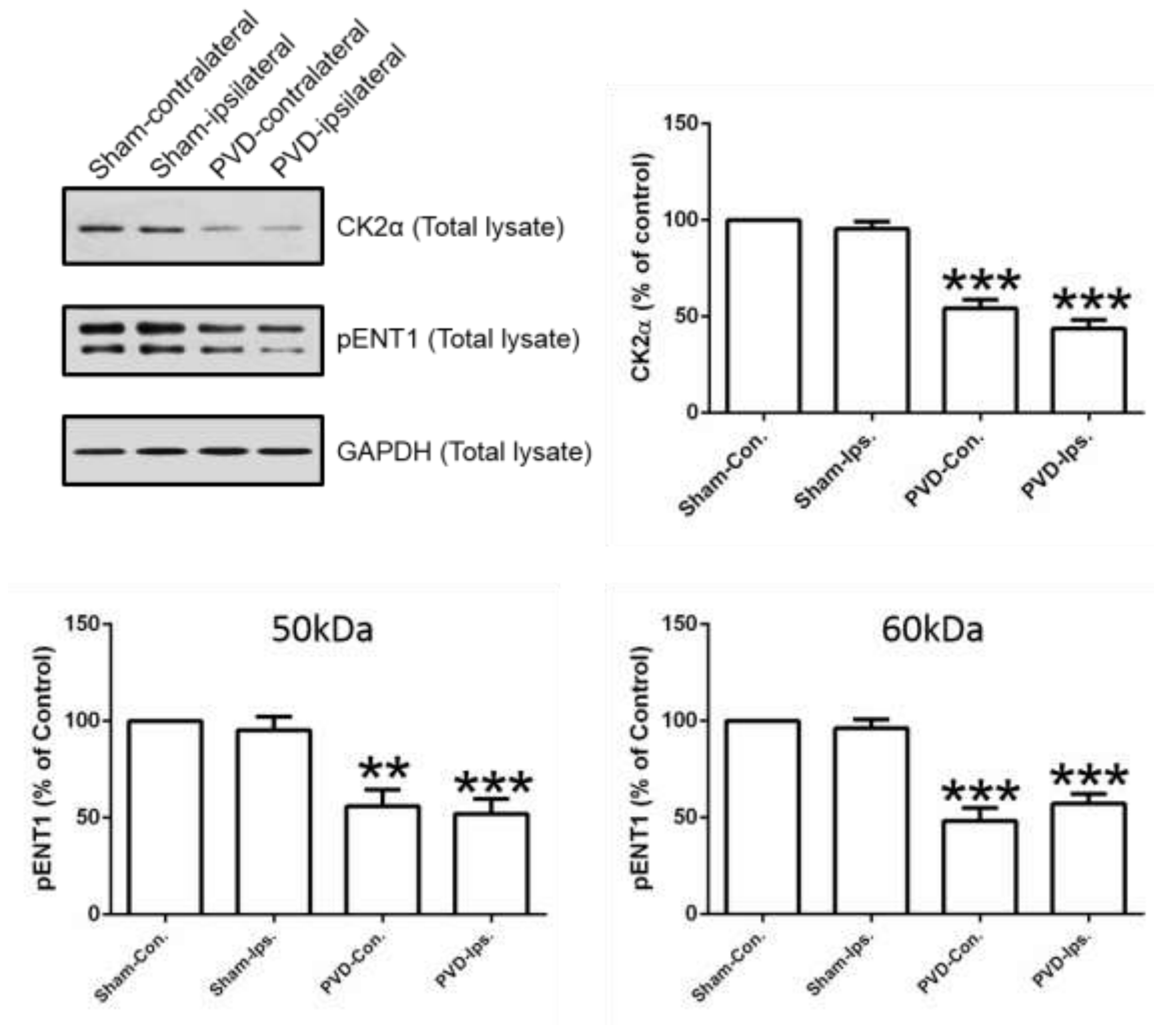


Figure 5.3 CK2α and pENT1 at Ser254 decreased in rat PVD model. After 48 hours of PVD stroke injury, total lysates of hippocampus were collected in Sham and PVD animals. The levels of CK2α and pENT1 at Ser254 were detected by western blotting. Both levels of CK2α and pENT1 at Ser254 decreased in the PVD model compared to Sham. (For description of our unilateral focal cortical PVD-induced stroke injury model, please refer to Chapter 2.)

are needed to determine whether the CK2 consensus site in GluA1 subunits shows decreased levels of phosphorylation after two days of PVD stroke injury. It is also important to determine whether this mechanism involving decreased CK2 levels during this advanced phase of ischemic damage contributes to neuroprotection of hippocampal neurons or represents a mechanism that attempts to reverse the effects of the acute CK2 activation in the early phase of hippocampal stroke damage. Moreover, future studies are also needed to address whether CK2 activation leads to activation p38 MAPK, JNK and PP2A, which leads to A1R-mediated synaptic depression and increased neurodegeneration (see summary in Figure 5.5B).

The potential sources of the extracellular adenosine in neurons and glial cells are the result of the activities of several adenosine-related proteins as shown in Figure 5.4. Briefly, the important proteins involved in adenosine generation include the following: 1) endo- and ecto-5'-nucleotidases that breaks down ATP to adenosine, 2) intracellular adenosine transport out of cells by adenosine transporters (ENT1 or *es*, and ENT2 or *ei*), 3) breakdown of cAMP to adenosine by cytosolic and ecto-phosphodiesterases, 4) inhibition of adenosine kinases (AK) leading to increased cytosolic adenosine, and 5) inhibition of intracellular adenosine deaminase, which also increases cytosolic adenosine. The extracellular concentration of adenosine is altered in response to environmental changes in the brain, such as during stroke or sleep. Due to the rapid metabolism of adenosine, it is difficult to detect the extracellular levels of adenosine, but there are some commercially available enzyme-based adenosine biosensors that indirectly measure adenosine levels in brain slices so that adenosine release can be correlated with synaptic depression in real time (Dale and Frenguelli 2009). However, I showed that the inhibition of CK2 can decrease the surface levels of ENTs and A1Rs. By this mechanism, I propose that CK2 normally promotes the surface expression of ENTs and A1Rs, thus optimizing the extracellular baseline levels of adenosine for optimal synaptic transmission. As previously reported, over-expression of ENT1 increased the volume of ischemic damage in the endothelin model of stroke (Soylu, Zhang et al. 2012). Here, we observed that the inhibition of CK2 decreased the phosphorylation and surface levels of ENT1. Taken together, these data suggests that the inhibition of CK2 leads to some form of an "ischemic preconditioning" in the brain which enhances the

Figure 5.4

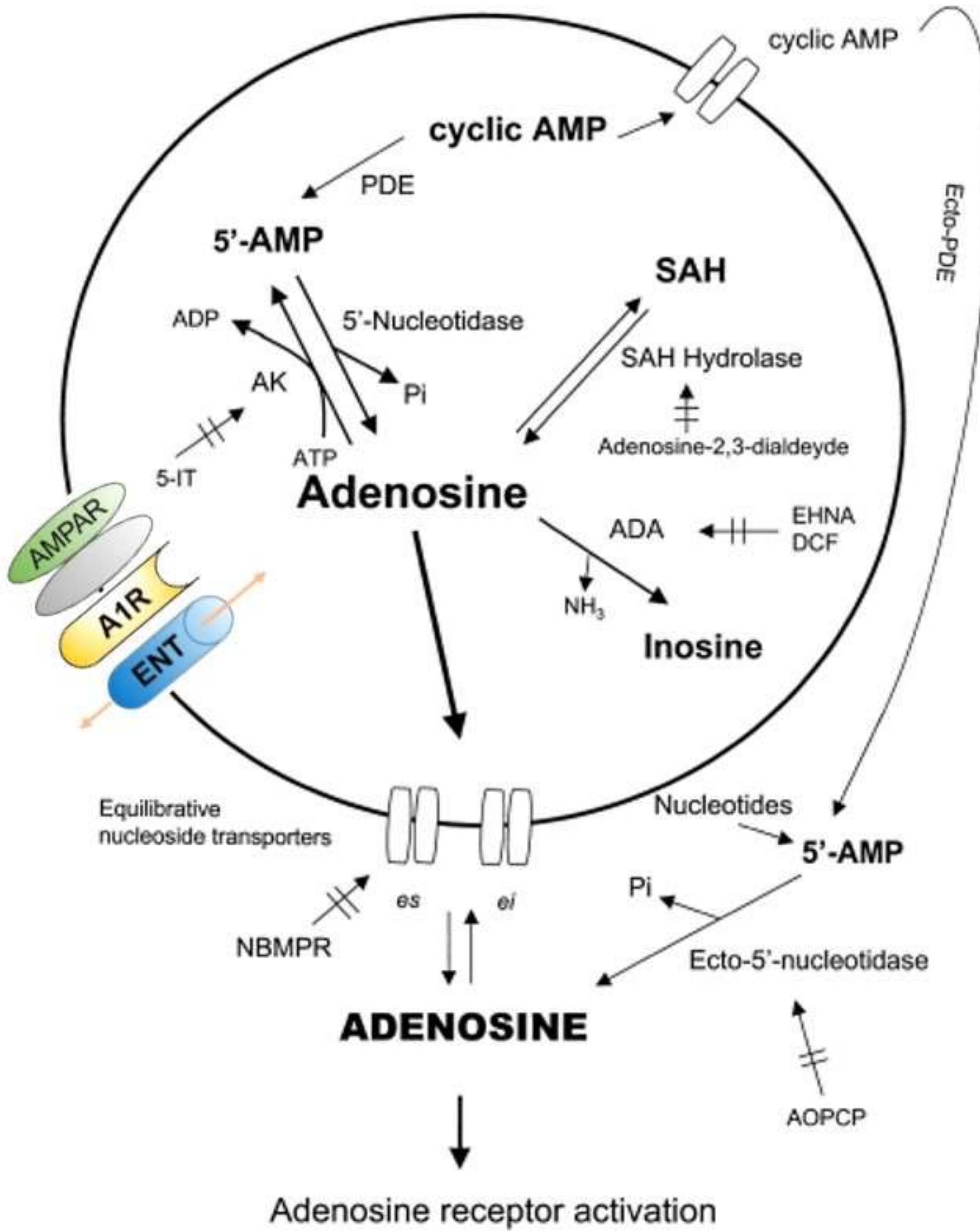


Figure 5.4 Pathways of adenosine production, metabolism and transport and effects of adenosine elevation on ENT-A1R-AMPAR signaling complex. Pharmacological inhibitors of specific enzymes are indicated. In ischemic brain cells, the elevation of extracellular adenosine is derived from various sources, including downregulation of ENT function, upregulation of ecto-5'-nucleotidase, decreased functions of cytosolic adenosine kinases and deaminases. Abbreviations are as follows: ADA, adenosine deaminase; AK, adenosine kinase; AOPCP, α,β-methylene ADP; DCF, deoxycoformycin; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine; *es*, equilibrative-sensitive nucleoside transporters; *ei*, equilibrative-insensitive nucleoside transporters; 5-IT, 5-iodotubercidin; NBMPR, nitrobenzylthioinosine; PDE, cAMP phosphodiesterase; SAH, S-adenosyl homocysteine. Modified from Latini and Pedata (Latini and Pedata 2001).

adenosine neuroprotective effects in subsequent ischemic insults, which was also confirmed by our *in vitro* hypoxia/reperfusion injury model and confocal images.

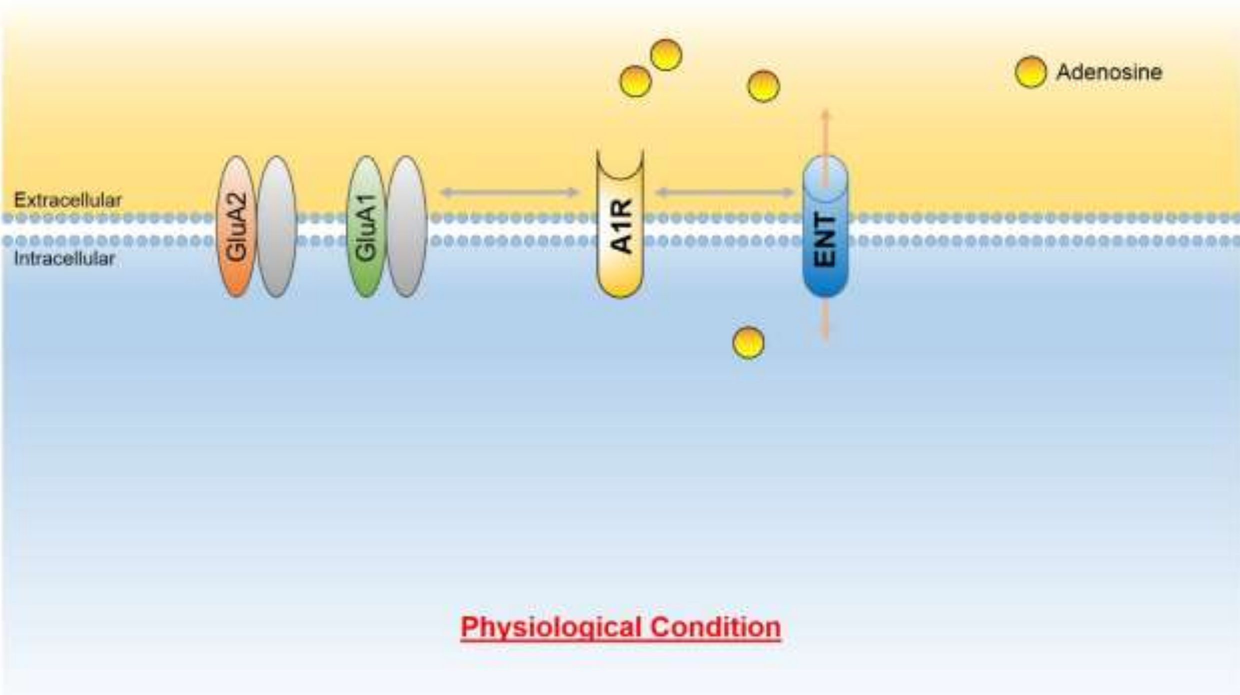
In Chapter 4, as AMPARs are known to play a crucial role in hippocampal synaptic plasticity and my data from Chapter 2 showed that elevation of adenosine levels in ischemic brains or prolonged, selective stimulation of A1Rs led to hippocampal synaptic depression, I therefore compared the levels of expression of AMPARs and adenosine receptors in membrane fractions and biotinylated (plasma membrane) fractions in the hippocampus of young and aged rats. In membrane fractions, we found that both GluA2 and GluA1-containing AMPARs were significantly reduced in 3, 6-7, and 10-12 month old rats compared to those in 1 month old rats. This was further confirmed with biotinylation assays that GluA2 and GluA1 surface expression was also significantly reduced in aged (10-12 month old rats) hippocampal tissue compared to younger (1 month old rats) tissue. Previously in Chapter 2, I found that A1R stimulation leads to profound synaptic depression. In addition, my data also support the involvement of GluA2 and GluA1 AMPAR endocytosis in aging process. Here, using biotinylation assays, I found that GluA2 and GluA1 surface levels were both significantly enhanced to similar levels in younger and aged hippocampal slices by chemical LTP. In contrast, recordings of fEPSPs in the CA1 region of the hippocampus showed higher levels of chemical LTP in younger versus aged hippocampal tissue. In addition, we found that Dynasore (endocytosis inhibitor) and Tat-GluA2-3Y peptide (inhibits clathrin-mediated endocytosis of GluA2), but not a scrambled Tat-peptide, caused significant enhancements of surface expressed GluA2 and GluA1 to similar levels after chemical induction of LTP in both young and aged brains. Since our biotinylation data do not distinguish between synaptic and extrasynaptic AMPARs that are being elevated during chemical LTP or with treatments with Tat-GluA2-3Y or Dynasore, our electrophysiological data do suggest, however, that there may be much higher levels of synaptic insertion of AMPARs during chemical LTP occurring in the young vs. aged hippocampal brains. Alternatively, our electrophysiological data may also suggest that the rate of AMPAR endocytosis may be increased in aged brains compared to younger brains, possibly owing to the increased adenosinergic signaling occurring in aged brains. Future studies into the activities and expression levels of the protein

machineries involved in clathrin-mediated endocytosis, including the role of ARC binding to dynamin 2 and endophilins 2 and 3 (Chowdhury, Shepherd et al. 2006), as well as studies into the expression levels and activities of MAPKs and protein phosphatases involved in clathrin-mediated endocytosis, may reveal differential expression levels of these components of endocytic machinery in young vs. older brains. Therefore, I propose that these potential changes that accompany increased adenosinergic signaling in aged brains may be important mechanisms that lead to increased endocytosis of AMPARs and impaired synaptic plasticity.

Since adenosine and adenosine receptors can regulate the release of excitatory transmitters (e.g. glutamate, acetylcholine, ATP) and alter the compositions and potential synaptic localizations of their respective receptors, the novel adenosinergic signaling mechanisms that I helped to unravel may have broader implications to several aging-related neurodegenerative diseases. Two decades ago, it was reported that a loss of A1R was observed in post-mortem tissue, including the hippocampus, from Alzheimer's disease patients (Kalaria, Sromek et al. 1990; Ulas, Brunner et al. 1993). This is followed by another interesting discovery of the role of adenosine signaling in Alzheimer's disease. In this study, A1R and β-amyloid colocalized in the post-mortem neocortical and hippocampal tissue from Alzheimer's disease patients (Angulo, Casado et al. 2003), suggesting adenosine receptors can be a valuable treatment target for Alzheimer's disease. The β-amyloid fragments have also been shown to bind to receptors to cause p38 MAPK activation (Munoz, Ralay Ranaivo et al. 2007; Munoz, Ramsay et al. 2010), and β-amyloid has also been shown to bind to AMPARs and promote their internalization (Zhao, Santini et al. 2010). Moreover, numerous G-protein coupled receptors, including the A2ARs, have been suggested to regulate the β-secretase (i.e., BACE1) expression and increase β-amyloid toxicity (Thathiah and De Strooper 2011). Therefore, based on my findings described in Chapters 2-4 and the strong links between adenosine signaling and β-amyloid toxicity described above, it is tempting to speculate that the new and revised mechanisms of the neurodegenerative signaling pathways that contribute to Alzheimer's disease

Figure 5.5

A



B

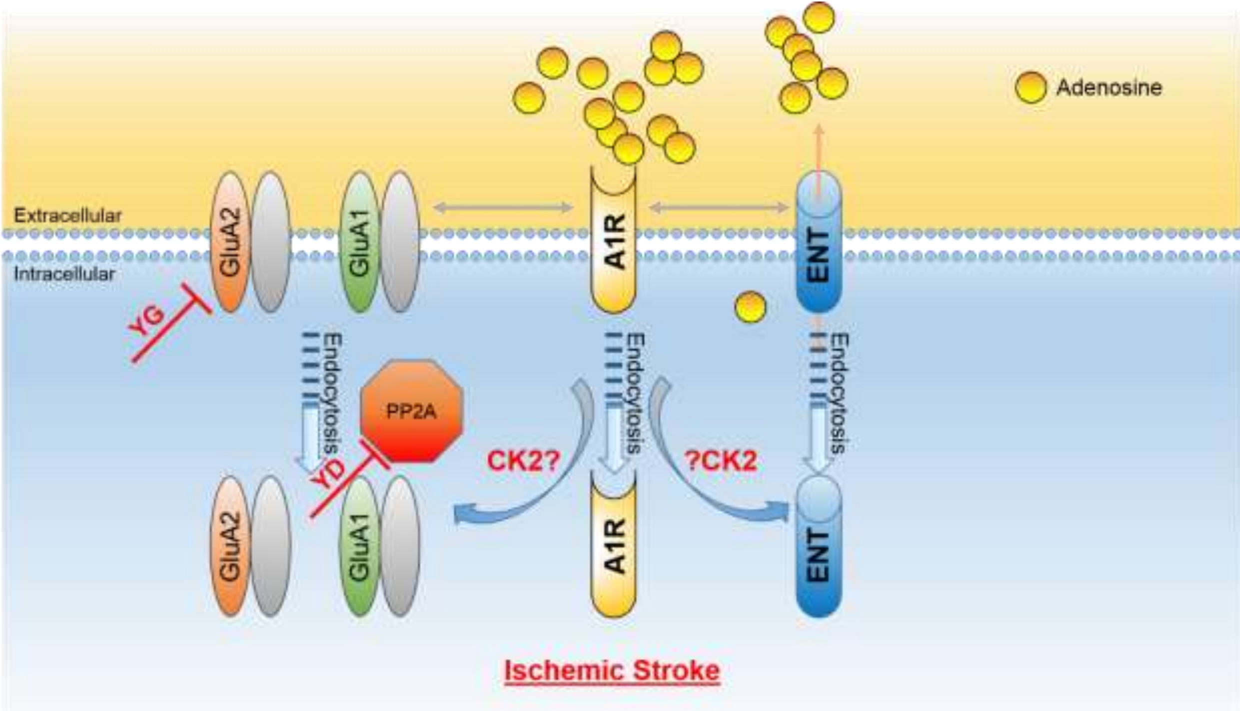


Figure 5.5 Proposed model for interaction between AMPAR-A1R-ENT protein complex: implication for stroke treatment. Activation of A1R leads to endocytosis of GluA2-containing AMPARs, which results in increased neurodegeneration. In contrast, stimulation of A1Rs could arise from increased adenosine tone, which results from decreased ENT surface expression following treatments with CK2 inhibitors. The accompanying decrease in A1R surface expression suggests that there would be decreased A1R-mediated adenosinergic signaling, which subsequently leads to decreased MAPK and PP2A activation. This proposed pathway could confer an "ischemic preconditioning" of hippocampal tissue, so that a subsequent ischemic injury would produce less neurodegeneration (B). Potential targets for neuroprotection using Tat-peptide interference strategies are indicated in B, where Tat-YG and Tat-YD prevents GluA2 endocytosis and PP2A activation, respectively.

pathogenesis may include A1R-induced AMPAR endocytosis (particularly of the GluA2 subunits) and increased β-amyloid-induced AMPAR endocytosis. It has been demonstrated that adenosine A2A receptor antagonist can prevent β-amyloid peptide-induced neuronal loss, indicating an existing relationship between adenosine signaling and Alzheimer's disease (Scatena, Martorana et al. 2007). However, further studies are needed to reveal the potential roles of both A2AR- and A1R-mediated signaling in the processing of amyloid precursor proteins and the β-amyloid toxic fragments generated by this proposed signaling in Alzheimer's disease brains.

Together, I have shown that a novel protein complex composed of AMPARs, A1Rs, and ENTs could facilitate the endocytosis trafficking of AMPARs, A1Rs and ENTs, which could be physiologically important in mediating neuronal responses to hippocampal ischemic damage. Also, the signaling mechanisms involved in AMPAR endocytosis, synaptic depression and neurodegeneration could very well be applied to other aging-related neurological diseases, where extracellular elevation of adenosine have been suggested, including Alzheimer's disease, Parkinson's disease and stroke.

5.2 Limitations

Adenosine receptors and AMPARs were confirmed to exist in the same protein complex. However, whether these two proteins interact directly or indirectly cannot be determined due to the limitation of the techniques I used, which included coimmunoprecipitation and colocalization. To resolve this problem, molecular biological techniques are needed to generate the potential domain-domain interactions in the two proteins. To do this, the entire sequences and parts of receptors are cloned and the resultant proteins should be expressed and purified to perform a "pull-down assay". It may be possible to tag one protein with a marker (e.g., by fusing a c-myc tag) and the other protein with a different type of marker (e.g., by fusing with a glutathione S-transferase or GST). Bioluminescence resonance energy transfer/fluorescence resonance energy transfer (BRET/FRET) or luciferase bioluminescence assays, which are robust and well established techniques, can be used for assessing direct protein-protein interactions in neurons.

Moreover, the binding abilities of adenosine receptors and the transport function of ENTs were not directly addressed in the present study. Due to the limitation of equipment, I only tested the surface levels of AMPA receptor, adenosine receptors and ENTs. However, it is known that the internalization of A1Rs causes the desensitization of A1R-mediated synaptic depression during hypoxia (Coelho, Rebola et al. 2006). Since the activation of A1Rs altered the surface level of ENTs and AMPARs, it is important to examine whether the prolonged activation of A1Rs leads to decreased rates of adenosine transport via ENTs or to increased calcium permeability of the resultant AMPARs, which are expected to be GluA2-deficient, and hence more calcium permeable. To directly measure the changes in adenosine transport rates, hippocampal synaptosomal membranes could be prepared after performing the required treatments (e.g., with CPA, CK2 inhibitors, ENT blockers, or vehicle controls) and loaded with ^{3}H-adenosine for scintillation counting, and the amount of radioactivity could reflect adenosine efflux from synaptosomes. To directly assess calcium influx through AMPARs, hippocampal neurons could be loaded with a calcium-sensitive dye or be transfected with a DNA plasmid that codes for the neuronal calcium sensor protein VILIP-1 (Braunewell, Brackmann et al. 2001), and then calcium signals will be imaged from neurons using live cell imaging with a confocal imaging microscope. The limitations in our studies could also be addressed in future by using genetically modified animals, for example, using ENT1 over-expressing animals (from FE Parkinson's lab, University of Manitoba), ENT1 knockout animals (from DS Choi's lab, Mayo Medical School), and A1R or A2AR knockout animals (from JF Chen's lab, Boston University).

The physical association between adenosine receptors and AMPARs does not even seem mandatory for the physiological/functional interaction to occur. No physical interaction was found either between A2ARs and AMPARs or between A2ARs and ENTs. Yet, the intracellular signaling pathways activated by A1Rs or A2ARs could be sufficient to produce changes in the trafficking or subcellular localizations of AMPARs. As shown in Chapter 2, the preincubation of A2AR agonist, CGS 21680, significantly increased the surface levels of GluA1 in cultured neurons. Moreover, Cunha's group has reported that A2AR played a predominant role in enhancing LTP (Rebola, Lujan et al. 2008; Costenla, Diogenes et al. 2011). Also, as we speculated above, CK2 may become activated after

A1R stimulation in the early phase of stroke damage, and later the CK2 levels could be downregulated during the later phase of stroke injury. A previous report has also described the negative regulation of A2ARs by CK2 (Rebholz, Nishi et al. 2009), and this was suggested in our studies, in that decreased CK2 levels would be expected to promote increased A2AR surface expression due to decreased A2AR desensitization from having reduced levels of CK2. Therefore, it is also possible that a signaling crosstalk (via CK2 activity/levels) exists between A1R and A2AR signaling, which could contribute to neurodegeneration in ischemic damage. Therefore, further investigations of the functional interactions between A2ARs/A1Rs and AMPARs are needed, which could provide further insight into the excitotoxic potential of adenosinergic signaling in stroke or other aging-related neurological diseases.

Another limitation is that DPCPX and Tat-GluA2-3Y peptide were not used in the PVD model. Given the ethical difficulty in deciding the right timing and dose needed to apply DPCPX or Tat-GluA2-3Y peptide to human stroke patients, preclinical animal experimental testing of these reagents is warranted. These additional studies will determine whether DPCPX or Tat-GluA2-3Y (both known to cross the blood-brain barrier) can prevent the PVD-induced decrease in the surface levels of GluA2 as well as the *ex vivo* electrophysiological changes (i.e., enhanced APSDs). Since AMPARs are essential for normal neuron functions, the clinical relevance of preventing AMPAR internalization, such as with the use of DPCPX or Tat-GluA2-3Y peptides during or after stroke attack, should be given more attention in future studies.

5.3 Future studies

As I mentioned above, due to limitations of coimmunoprecipitation and colocalization in this study, I could not determine whether adenosine receptors and AMPARs interact directly. Full-length or partial fragments of purified A1Rs and AMPARs are needed to perform pull-down experiment to test whether they directly associate.

Since the calcium permeability of AMPARs is important for neuronal excitation, calcium imaging can be applied to test calcium permeability of AMPAR after the activation of adenosine A1 receptor.

It is known that AMPAR trafficking is crucial for neuronal functions, including LTP and LTD. Therefore, aberrant trafficking of AMPARs induced by excessive activation of A1Rs can cause dysfunction of neurons leading to neuronal damage and death. Consequently, whether the prolong activation of A1Rs can induce neuronal apoptosis or neuronal death needs further studies.

The scaffolding and endocytic protein-family of β-arrestins facilitates G-protein-coupled receptor (GPCR)-stimulated MAP kinase activation and internalization (Luttrell and Lefkowitz 2002). Arrestin regulates A1R by decreasing the plasma membrane level of A1R (Jajoo, Mukherjea et al. 2010). Therefore, arrestin may play a role in A1R-induced endocytosis of AMPARs. Future studies are needed to test whether the arrestin-family is also involved in the endocytosis and recycling of A1Rs to neuronal plasma membranes.

As we reported above, prolonged activation of A1Rs induces increased AMPAR endocytosis, and this could be one important mechanism underlying post-stroke injury. Therefore, interruption of the putative interactions between A1Rs and AMPARs could be neuroprotective to the post-stroke brain. To develop such a therapy, further studies are needed for defining the regions of interactions between A1Rs and AMPARs. Such interactions should include determining whether they directly interact, as well as by determining where the domain-domain interactions are occurring in these two receptors. Drugs, e.g. peptides, can be subsequently designed to interrupt the interaction in order to minimize post-stroke neuronal damage. Lastly, further experimentation is required to confirm whether an increase in extracellular adenosine concentrations after CK2 inhibition can alter synaptic transmission and neuronal health. Preliminary studies, as described earlier, do indicate that CK2 inhibition does lead to increased adenosine tone, which has been inferred from electrophysiological recordings showing increased synaptic depression.

5.4 Conclusions

The goal of the current study is to provide a comprehensive view of adenosinergic signaling. I established that A1Rs and GluA2-containing AMPARs formed structurally stable protein complexes in hippocampus. Novel functional association between A1R and

AMPARs was also determined. Additionally, prolonged stimulation of A1Rs with the agonist CPA was found to cause APSD in hippocampal brain slices, and APSD levels were blunted by inhibiting clathrin-mediated endocytosis of GluA2 with the Tat-GluA2-3Y peptide. On the other hand, prolonged CPA incubation showed a significant depletion of GluA2 and GluA1 surface expression from hippocampal brain slices and cultured hippocampal neurons. We also found that activation of A1R-induced internalization is through clathrin-mediated endocytosis pathway. In addition, A1R-mediated internalization of GluA2, but not GluA1 subunits, is p38 MAPK- and JNK- dependent. Lastly, we showed that a focal cortical ischemia in an *in vivo* small-vessel stroke model altered hippocampal surface expression of AMPARs and adenosine receptors that contributes to tonic synaptic depression, indicating A1R-mediated internalization of AMPARs is a mechanism that mediates post-ischemic neuronal damage.

My study also provides novel evidence that ENTs are structurally coupled with A1Rs, and this coupling is involved in the CK2-mediated cell surface trafficking of ENT1 and ENT2 in the rat hippocampus. Our data also showed that during hypoxia the CK2 inhibitors caused a more dramatic inhibition of surface localization of ENT1 and A1Rs, implying that these changes can potentially exacerbate ischemia-induced neuronal damage. Therefore, my studies suggest that these CK2-mediated changes in ENT and A1R surface expression represent an important mechanism for cerebral ischemic damage, but future studies are needed to determine whether activation of CK2 and consequently increased surface trafficking of ENTs, are important for neuroprotection.

After studying adenosinergic signaling under pathological hypoxic/ischemic condition, adenosinergic signaling in aging brains was also investigated, given the high prevalence of aging-related disorders in the elderly, such as stroke and other memory deficits. We studied surface levels of GluA1- and GluA2-containing AMPARs in aged animals and found that both AMPAR subunits were decreased during aging. Neuron excitatory level is also reduced during aging as recordings of fEPSPs in the CA1 region of the hippocampus showed lower levels of chemical LTP in aged versus young hippocampal tissue. Moreover, I found that Dynasore (endocytosis inhibitor) and Tat-GluA2-3Y peptide (inhibits clathrin-mediated endocytosis of GluA2), but not a scrambled Tat-peptide, enhanced the surface expression of GluA2 and GluA1 after chemical

induction of LTP in both young and aged brains. Therefore, these results indicate that increased adenosinergic signaling in aged brains leads to increased endocytosis of AMPARs and impaired synaptic plasticity.

Taken together, these data suggest that multi-protein complexes composed of AMPARs, A1Rs, and ENTs in the hippocampus are crucial for regulating the functions of adenosine under ischemic condition. Endocytosis of AMPARs induced by A1Rs can cause neuronal damage. Therefore, preventing the A1R-induced endocytosis of AMPARs is a novel approach to minimize the neuronal damage caused by stroke. Since CK2 also regulates adenosine receptors and transporters, CK2 inhibitors may be very effective in attenuating neuronal damage caused by a subsequent hypoxic insult. In conclusion, results from my studies have unraveled novel mechanisms of adenosinergic signaling, which could promote excitotoxicity in ischemic brain damage. These novel mechanisms provide a rich opportunity for designing alternative and novel neuroprotective therapies to combat the well-known phenomenon of delayed neuronal cell death that occurs days and even weeks after the initial ischemic stroke injury. This will ultimately benefit the over fifty-thousand Canadians who suffer a stroke every year, as well as the aging population which presents with increased incidence of aging-related neurological diseases, including stroke.

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