Adenosine Signalling and Regulation of Ca^{2+}-permeable AMPA Receptors in Hypoxia

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Abstract

Stroke is one of major causes of death, and ischemic stroke attributes to approximately 87% of all strokes. Our lab has previously shown that during an ischemic insult, adenosine A1 receptor (A1R) activation in the rat hippocampus leads to AMPA receptor (AMPAR) downregulation and persistent synaptic inhibition. This persistent synaptic depression could contribute to neuronal damage, as neurons require constant excitatory inputs. Moreover, we observed an adenosine-induced post-hypoxia synaptic potentiation (APSP) in rat hippocampal CA1 field potential recordings. Hypoxia-induced cell death and APSP were significantly reduced when rat hippocampal slices were pretreated with either A1R or A2AR antagonist, indicating a potential interaction between A1Rs and A2ARs through yet unknown mechanisms. This study further explores the role of glutamate receptors in the generation of APSP through adenosine signalling. We hypothesize that hypoxia induces A1R-mediated, dynamin (a GTPase regulating endocytosis)-dependent internalization of A1Rs and GluA2/GluA1 AMPARs, which is then followed by an A2AR-mediated upregulation of these AMPARs, which is a prerequisite for full expression of APSP. Electrophysiology studies demonstrated that synaptic transmissions and APSPs in the rat hippocampal CA1 region are mostly mediated by AMPAergic mechanisms, instead of NMDA receptors, and that these required both A1R and A2AR signalling cross-talk. To determine whether Ca^{2+}-permeable AMPARs (CP-AMPARs) underlie APSP, I performed experiments to test the effects of selective CP-AMPAR antagonists on APSP levels, including NASPM, IEM 1460 and Philanthotoxin-74. Application of the CP-AMPARs antagonists at the early phase of hypoxic stimulation blocked the generation of APSPs, whereas no attenuated effects were observed when applied after the expression of APSPs. In contrast, the clinically approved anti-seizure drug Perampanel, which is a non-competitive AMPAR antagonist, blocked the generation of APSP. Surprisingly, all CP-AMPAR antagonists tested were effective in preventing hypoxia-induced hippocampal neuronal damage during the early phase of hypoxic stimulation, but Perampanel was the only compound that prevented neuronal damage during normoxic brain slice reperfusion. Additional studies of leukocyte-specific protein1 (LSP1) knockout mice also revealed a potential contribution of LSP1 to altering synaptic plasticity during hypoxia-reperfusion injury models. In particular, LSP1 may regulate the levels

of synaptic depression and CP-AMPARs during A1R stimulation in hypoxic conditions. Collectively, this study has provided further evidence for CP-AMPARs' role in delayed hippocampal injury, which involves both A1Rs and A2ARs, and reveals GluA2-lacking AMPARs as a potential target for designing neuroprotective drugs in the late stage neurodegeneration related to hypoxia/ischemia.

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AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APSP adenosine-induced post-hypoxia synaptic potentiation

A1R adenosine A1 receptor

A2AR adenosine A2A receptor

cLTP chemically induced long-term potentiation

CNQX 6-cyano-7-nitroquinoxaline-2,3-dione

CPA N^6-Cyclopentyladenosine

DMSO Dimethyl sulfoxide

DNQX 6,7-dinitroquinoxaline-2,3-dione

DPCPX 8-Cyclopentyl-1,3-dipropylxanthine

fEPSP field excitatory post synaptic potential

IEM 1460 N,N,H,-Trimethyl-5-[(tricyclo[3.3.1.13,7]dec-1-ylmethyl)amino]-1-pentanaminiumbromide hydrobromide

LSP-1 Leukocyte-specific protein 1

MK-801 (5R,10S)-(-)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cylcohepten-5,10-imine maleate

NASPM 1-Naphthyl acetyl spermine trihydrochloride

NBQX 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide

NMDAR N-methyl-D-aspartate receptor

PVD pial vessel disruption

YG Tat-GluA2–3Y

Introduction

1.1 Hypoxia and Ischemic Stroke

There are approximately 62,000 strokes occurring in Canada each year, and almost half of Canadians either experienced it themselves or have had a close family member or friend who suffered from a stroke. More than 400,000 Canadians experience long-term disability from stroke, and this number will likely double in the next 20 years (Stroke Report 2017). Ischemic stroke is a common form which accounts for about 80% of all strokes. Hypoxia has been frequently observed after stroke and it leads to multiple detrimental outcomes. Severe hypoxia leads to rapid decrease in oxygen level, which can result in permanent damage to the neuronal system in a short period of time (Ferdinand and Roffe 2016). Observations in clinical trials have shown that hypoxia in the brain can cause short-term memory deficits, difficulty in judgment and inability to accomplish complex tasks (Cooper et al. 2015; Komiyama et al. 2015).Long-term influence of hypoxia are more widespread in the brain and should be taken into serious consideration. In older adults, hypoxic injury in the basal ganglia can result in seizures and movement disorders (Howard et al. 2011). Brain hypoxia-ischemia is now a common disorder along with a high probability of morbidity in the elderly, often resulting in severe stroke (Hung et al. 2017). Functional outcomes of ischemic injury in the brain is not predicted well compared to other tissues. Although current knowledge of the biochemical and physiological bases of the hypoxia-induced brain damage is well studied, the reason behind this unique vulnerability of the brain to stroke still warrants further investigations (Gooshe et al. 2015; Payabvash et al. 2011). The human brain is specifically vulnerable to ischemic insults since it is an intrinsically, metabolically active organ yet contains virtually no O_2 reserve. One of the early consequence of hypoxia-ischemia is energy failure, which leads to abnormal ion channel activities. Decrease of Na^+ gradients after hypoxia will trigger accumulation of extracellular glutamate, which will result in overstimulating glutamate receptors and neurodegeneration (Dong et al. 2009; Lai et al. 2014; Ribeiro et al. 2010; Schauwecker 2010).

1.2 Stroke and Adenosine

Adenosine has a crucial role in the central nervous system (CNS). There is an inhibitory effect of adenosine on the neurotransmission and plays a neuroprotective role to the CNS in certain conditions (Sperlágh and Vizi 2011). Therefore, the importance of studying the generation and release of adenosine in the human brain has been highlighted by neuroscientists. As an endogenous purine nucleoside, adenosine showed its significance in regulating multiple physiological processes, such as promoting sleep and suppressing arousal (Huang et al. 2014; Maximino et al. 2011; Porkka-Heiskanen and Kalinchuk 2011). In the healthy brain, the expression of extracellular adenosine is relatively low, but rapidly increases following hypoxic-ischemic insult *in vitro*. For example, in the rat stroke model when animal suffered from short period ischemia (10 to 15 minutes), the level of extracellular adenosine in the rat hippocampal slices are accumulated by around 20 μM (Latini et al. 1999). Electrophysiological studies in the hippocampal slices have shown that adenosine inhibit neurotransmission in the rat CA1 region and neuronal excitability was also attenuated (Sperlágh and Vizi 2011). In early studies, scientist believed that adenosine and adenosine A1 and A2A receptors could develop into potential therapeutic targets for stroke patients (Kitagawa et al. 2002; von Lubitz 1999; Pedata et al. 2007). However, considering the large scale of the expression of adenosine receptors in the CNS and their overall effect on the human body, adenosine-based therapies have encountered a great deal of difficulties. Although some studies have observed that early application of adenosine (i.e., at the time of stroke) reduces neuronal damage, clinical administration of adenosine could cause decreased blood pressure and respiratory alkalosis (Kitagawa et al. 2002; Layland et al. 2014). Even with the observation that adenosine may have neuroprotective effects in animal models, accurate administration is still a prerequisite since adenosine has a short window of efficacy. Additionally, adenosine has a comparable short half-life (less than 15 seconds) in human plasma and it will decrease blood brain barrier permeability (Pardridge et al. 1994), which presents great difficulties in utilizing adenosine in targeted delivery during clinical applications. Finally, administration of adenosine has opposing effects on different adenosine receptors, due to their different functions and diverse distribution in the CNS, which brings more difficulties to its application in clinical trials.

1.2.1 Adenosine and Adenosine Receptors

As an important neuroeffector, the functions of adenosine in the CNS are regulated by several adenosine receptors. There are four types of G-protein coupled receptors in the adenosine receptor family, with the names of A1, A2A, A2B, and A3 receptors (A1Rs, A2ARs, A2BRs, and A3Rs). A1 and A3 were found to couple to inhibitory G proteins, whereas A2A and A2B were found to couple to stimulatory G proteins (Sheth et al. 2014). Among the four receptors, A1R is not only the most widely expressed receptor in the brain, but also has the highest affinity for adenosine (Ribeiro and Sebastio 2010). Highly expressed A1R contributes to the reduction of neurotransmission through elevated adenosine in the hippocampus and regulates many aspects of the physiological processes involved in neuronal excitability and synaptic plasticity. A1R activation has shown to be controversial in both *in vivo* and *in vitro* animal models of ischemic strokes (Manjunath and Sakhare 2009). Some studies using A1R knockout mice showed no significant alteration in neuronal damage induced by global ischemia when compared to studies of wild-type mice. Additionally, pre-treatment of A1R antagonist 8-CPT at the onset of global ischemia aggravated damage (Olsson et al. 2004). However, our lab showed that in hippocampal slices, decreased synaptic transmission and neurodegeneration in the CA1 region caused by 20-min hypoxia treatment were attenuated by the application of A1R antagonist DPCPX (Stockwell et al. 2016). Moreover, DPCPX also showed a beneficial effect during the recovery period after the treatment of hypoxia or prior to administration of a selective A1R agonist *in vivo* (Stockwell et al. 2016, 2017). These results indicated that A1R antagonists could have a detrimental or beneficial effect in hypoxia at the neuronal level depending on the time of administration (i.e., chronic *vs.* acute). Although some studies suggested that acute stimulation of A1R has neuroprotective effect by decreasing synaptic transmission (Williams-Karnesky and Stenzel-Poore 2009), chronic downregulation of A1R during post-stroke in animal stroke model or after chronic A1R-agonist stimulation has been proven to be contributory for the ischemia-induced insults and impaired long term potentiation (LTP) (Chen et al. 2014, 2016). A2ARs are also widespread in the brain (concentrated in basal ganglia) with a lower density compared to A1R; it also has a relatively high affinity for adenosine. In opposition to A1R, up-regulation of A2ARs are observed in brain after ischemia

and researches have justified the importance of A2AR antagonists as potential neuroprotective treatment in ischemic stroke, Alzheimer's and Parkinson's disease (Casetta et al. 2014; de Lera Ruiz et al. 2014; Paterniti et al. 2011; Pedata et al. 2007; Reyhani-Rad and Mahmoudi 2016; Stockwell et al. 2017). Since A2BRs and A3Rs have low abundance in the brain (Garcia et al. 2014), the role of these two receptors have received considerably less attention.

1.2.2 Potential Cross Talk Between Adenosine A1 and A2A receptors

The mechanism by which adenosine modulates synaptic transmission in hypoxic/ischemic injury remains poorly resolved. Elevation of extracellular adenosine in the hippocampus during ischemia or hypoxia stimulates both A1Rs and A2ARs, and adenosine-mediated neuroprotection or neurodegeneration is due to A1R and A2AR stimulation, respectively (Stockwell et al. 2017). A1R activation inhibited glutamatergic synaptic transmission mainly through presynaptic inhibition of glutamate release, while A2ARs have been shown to facilitate glutamatergic synaptic transmission. Therefore, some reports in the past suggested that stimulating A1R and inhibiting A2AR may have neuroprotective effects during ischemia (Sweeney 1997; Thauerer et al. 2012). Although at the onset of neuronal injury, A1R activation attenuates brain damage, A1R is downregulated in chronic noxious situations. Conversely, A2ARs are up-regulated in noxious brain conditions (Cunha 2005) and A2AR antagonists have proved to be potential neuroprotective agents in multiple neurodegenerative diseases (Casetta et al. 2014; de Lera Ruiz et al. 2014; Pedata et al. 2007; Reyhani-Rad and Mahmoudi 2016). Our lab reported that hippocampal A1Rs and excitatory AMPA receptors (AMPARs) are downregulated while A2ARs are upregulated after a 20-min hypoxic insult or focal cortical ischemia using a pial vessel disruption (PVD) procedure (Chen et al. 2014; Stockwell et al. 2016). Moreover, A1Rs and AMPARs are physically coupled, whereas A2ARs and AMPARs are not (Chen et al. 2014). Following hypoxia during normoxic washout, we observed A2AR-dependent synaptic potentiation (Stockwell and Cayabyab, unpublished) and therefore hypothesized that A1R stimulation is required for subsequent adenosine-induced post-hypoxia synaptic potentiation (APSP). Specifically, we found that a 20-min hypoxia treatment followed by 45-min normoxic washout/reperfusion produced elevated APSP (150-160%), which was

abolished by pre-treatment with either DPCPX (100 nM), an A1R antagonist, or SCH 442416 (5 nM), an A2AR antagonist. Accordingly, using propidium iodide to label damaged cells, hypoxia alone caused significant neuronal death, which was significantly reduced by pre-incubation in either DPCPX or SCH 442416. These results suggest that both A1R and A2AR are involved in the APSP generation we observed, as well as the neurodegeneration caused by hypoxia treatment in the rat hippocampal CA1 region, suggesting that the traditional idea of A1Rs and A2ARs as individual parallel signalling systems need to be revised.

1.3 Stroke and Glutamate receptors

Excitotoxicity is a leading cause of central neuronal loss in noxious brain conditions. After global ischemia, elevated extracellular glutamate was observed in the CNS, which is believed to be important to the biological processes which leads to the impairment of post-hypoxia brain tissue. A few glutamate receptors are discovered to regulate this excitotoxicity, including NMDA and AMPA receptors (NMDARs, AMPARs) as well as kainate receptors.

At the early stage of hypoxia, excitotoxicity and the excessive load of calcium are two main factors towards neurodegeneration. (Annunziato et al. 2007). Glutamate, serves as the most widely expressed neurotransmitter, accumulates in the extracellular space due to energy and ion pump failure.

The accumulation of glutamate triggers prolonged stimulation of AMPARs and NMDARs, which will result in dramatic enhancement of Ca^{2+} and Na^{+} influx in neurons. In studies using rat models of several neurological disorders, such as epilepsy, ischemic stroke and Parkinson's Disease, scientists believed that glutamatergic synapse is the potential site for neuroprotective drugs to target. (Kostandy 2012; Lai et al. 2014; Schauwecker 2010; Traynelis et al. 2010). Although scientists have believed that NMDAR and AMPAR antagonists have neuroprotective effects in animal ischemic stroke models for nearly three decades (Meldrum 1990), numerous clinical trials targeting NMDARs and AMPARs have been disappointing in ischemic stroke patients (Ginsberg 2009; Hoyte et al. 2004; Ikonomidou and Turski 2002). Preclinical data have suggested that protection of neurons induced by NMDAR antagonists is most effective if they

are applied near the onset of the ischemic stroke. If the antagonists were applied after the onset, the neuroprotective effect will be diminished with time. The protective effects usually disappears at 1.5-2 hours after the onset (Lipton 2004). NMDAR antagonist like Selfotel has been proved that it has little neuroprotective towards acute ischemic stroke. Moreover, if Selfotel were applied in the first month on patients with severe ischemic stroke, it might lead to a higher rate of death. (Davis et al. 2000).Clinical trials with the application NMDAR antagonists in ischemic stroke and brain injury have shown little therapeutic benefit (Hoyte et al. 2004; Ikonomidou and Turski 2002; Moretti et al. 2015; Roesler et al. 2003). AMPAR antagonists were also proposed to be useful neuroprotective drugs (Constals et al. 2015; Frampton 2015; Nayak and Kerr 2013; Whitehead et al. 2017). Competitive AMPAR blockers such as 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)qui-noxaline (NBQX), showed robust neuroprotection in several animal stroke models (Filliat et al. 1998; Meden et al. 1993). Nonetheless, due to their side effects and difficulty in drug delivery, their applications in clinical trials have also been disappointing (Buchan et al. 1993).

1.3.1 AMPA receptors

AMPA receptor is an important inotropic glutamate receptor in the CNS. AMPARs express in the neurons and glial cells, which mainly regulates fast-excitatory synaptic transmission (Traynelis et al. 2010). There are four subunits for assembling AMPARs, GluA1, GluA2, GluA3 and GluA4. In the hippocampal CA1 regions, the AMPARs containing GluA1-GluA2 heteromers are most abundant (Lu et al. 2009). CA1 region is crucial to memory and the induction of LTP is mainly regulated by phosphorylation of GluA1 during the recruitment of AMPARs to the synapses (Zamanillo et al. 1999). Meanwhile, GluA2 is regulating AMPAR endocytosis and long-term depression (LTD). Also, if an AMPAR contains at least one GluA2 subunit, it will prevent calcium form entering the cells. Although a large portion of AMPAR in the CNS is GluA2-containing AMPARs, GluA2-lacking AMPAR has also shown its importance in the hippocampus, habenula and cortex (Wright and Vissel 2012). Since AMPARs mainly regulates fast synaptic neurotransmission in the hippocampus, the precise regulation of AMPARs has proved to be important in investigating synaptic plasticity and neurotransmission.

Multiple studies on the regulation of GluA2-lacking AMPARs have revealed their importance in neurological diseases such as ischemic stroke, epilepsy, Parkinson's and Alzheimer's Disease (Berridge 2011; Chang et al. 2012; Gomes et al. 2011; Weiss 2011; Whitehead et al. 2017). During ischemic stroke, GluA2 is downregulated, which leads to AMPAR-regulated accumulation of calcium and zinc influx (Noh et al. 2005). Studies on the effects of GluA2-lacking AMPAR antagonists have shown reductions of post-ischemic Zn^{2+} accumulation and neuronal death in the brain (Liu et al. 2004), which demonstrated the importance of GluA2-lacking AMPARs in ischemic stroke. Some preclinical data indicated that AMPA/kainate receptor blockers have better neuroprotective effects when compared to NMDAR blockers during ischemic conditions (Dhawan et al. 2011; Ikonomidou and Turski 2002; Lau and Tymianski 2010; Traynelis et al. 2010). The neuroprotective effect might due to the blockade of GluA2-lacking AMPARs, which indicates that the presence of GluA2-lacking AMPARs are associated with this hypoxia induced neurodegeneration (Talos et al. 2006).

1.3.2 NMDA receptors

N-methyl-D-aspartate receptor (NMDAR) is another important receptor which regulates neurotransmission and synaptic plasticity in the CNS. NMDARs contains three kinds of subunits, GluN1, GluN2 and GluN3 (Traynelis et al. 2010). Similar to AMPAR, NMDAR is another crucial Ca^{2+}-permeable receptor implicated in the process of excitotoxicity. Early studies have suggested that blockade NMDA receptor may protect neurons during ischemic insults (Ikonomidou and Turski 2002; Meldrum 1990; Simon et al. 1984), which intrigued neurologists to seek out solutions for ischemic stroke from NMDAR antagonists. During ischemic stroke, large Ca^{2+} influx occurs in the hippocampal CA1 region, which leads to neurodegeneration and cell death (Tymianski and Tator 1996). Administration of MK-801, a NMDA receptor antagonist, attenuates Ca^{2+} influx during ischemia, however, has little effect in preventing cytotoxic intracellular rises in Ca^{2+} concentrations during post-ischemia period (Silver and Erecińska 1992). After the onset of hypoxia induced stroke, glutamate is accumulated on the brain, however, the damage could last for days. This might explain the failure of NMDAR antagonist based drugs since they are ineffective in the delay

neurodegeneration (Albers et al. 1999; Davis et al. 2000). Also, more focus on non-glutamatergic-induced mechanisms of neurotoxicity warrant further investigation.

1.3.3 GABA receptors

Gamma-amino- butyric acid (GABA) mediates inhibitory neurotransmission in the CNS and acts by reducing the depolarization-induced and ischemia-induced glutamate release. There are two major subtypes of GABA receptors, including the anion channels $GABA_A$ and presynaptic G-protein coupled receptors $GABA_B$. In both *in vivo* and *in vitro* animal stroke models, sciencists have shown that elevation of extracellular GABA levels leads to reduction of $GABA_A$ receptor density (Hiu et al. 2016; Schwartz-Bloom and Sah 2001). In multiple clinical trials, drugs based on GABAergic mechanisms have shown to be successful on protecting the brain in several neurologic diseases, such as anxiety disorders, depression, seizures, Parkinson's disease and Alzheimer's disease (Greenfield 2013; Jo et al. 2014; Luscher, Fuchs, and Kilpatrick 2011; Rudolph and Möhler 2014; Vithlani et al. 2011).

1.4 Therapeutic targets for ischemic stroke

1.4.1a AMPA receptors

The application of AMPAR antagonists in potential stroke treatments are divided into two classes: competitive agents and non-competitive agents. On the one hand, NBQX and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) are widely used competitive agents for blocking AMPARs. GYKI 52466, on the other hand, serves as a commonly applied non-competitive agent. However, several reports in the literature highlight the controversial results on the neuroprotective effects of selective AMPA receptor antagonists (Nayak and Kerr 2013; Schauwecker 2010; Schielke et al. 1999). Brickley's lab discovered that CNQX could increase GABA transmission in the cerebellum, indicating these agents might affect synaptic transmission by a non-AMPA mechanism(Brickley et al. 2001). Although NBQX has shown protective effects in reducing neuronal cell death in the hippocampal region, it has been suggested that treatment with NBQX was ineffective in reducing neurodegeneration in rat

stroke models (Meden et al. 1993). Since NBQX has a very short half-life (Ikonomidou et al. 1996) when applied at relatively high dose in order to provide neuroprotection and it has a low solublity in water, therefore, it is not ideal for administration in human patients. Some studies on rat stroke model suggested that GYKI 52466 was effective in neuroprotection; however, the drug delivery to patients is complicate and it has side effects including hypothermia (Nayak and Kerr 2013).

1.4.1b Ca^{2+}-permeable AMPARs

Studies have suggested that high ischemic susceptibility corresponds to increased Ca^{2+}-permeable AMPAR numbers, indicating that GluA2-lacking AMPARs are associated with hypoxia induced neuronal damage (Talos et al. 2006). In rat hippocampus, pyramidal neurons in the CA1 region are specially vulnerable to ischemic injury. Evidences have been provided showing that increased numbers of Ca^{2+}-permeable AMPARs are related to delayed cell death in the rat hippocampal CA1 region (Anzai et al. 2003; Noh et al. 2005; Weiss 2011). In addition, neuronal injury was attenuated when Ca^{2+}-permeable AMPAR blockers were applied after the ischemic insult has occurred (Noh et al. 2005). All in all, these results prompted our lab to further investigate Ca^{2+}-permeable AMPARs as a therapeutic target for ischemic stroke.

1.4.2 Perampanel

Perampanel, also referred to by its trademarked name Fycompa, is a non-competitive AMPAR antagonist. The Food and Drug Administration (FDA) has approved Perampanel as the first orally active drug in patients with epilepsy for patients older than 12 (Rohracher et al. 2015). Similar to other AMPA receptor antagonists, preclinical studies of Perampanel have identified its broad-spectrum anti-seizure effects in acute seizure models (Frampton 2015; Patel 2015; Rogawski and Hanada 2013). However, apart from other AMPAR antagonists, Perampanel has a distinctively very long half-life in humans, with gradual accumulation in plasma that could contribute to the development of tolerance or desensitization-related effects. Since Perampanel is already FDA-approved for seizures, this encouraged our lab to further investigate if repurposing this drug for other neurological diseases, such as ischemic stroke, Alzheimer's

disease and Parkinson's disease, could provide an alternative neuroprotective therapy in preclinical animal studies.

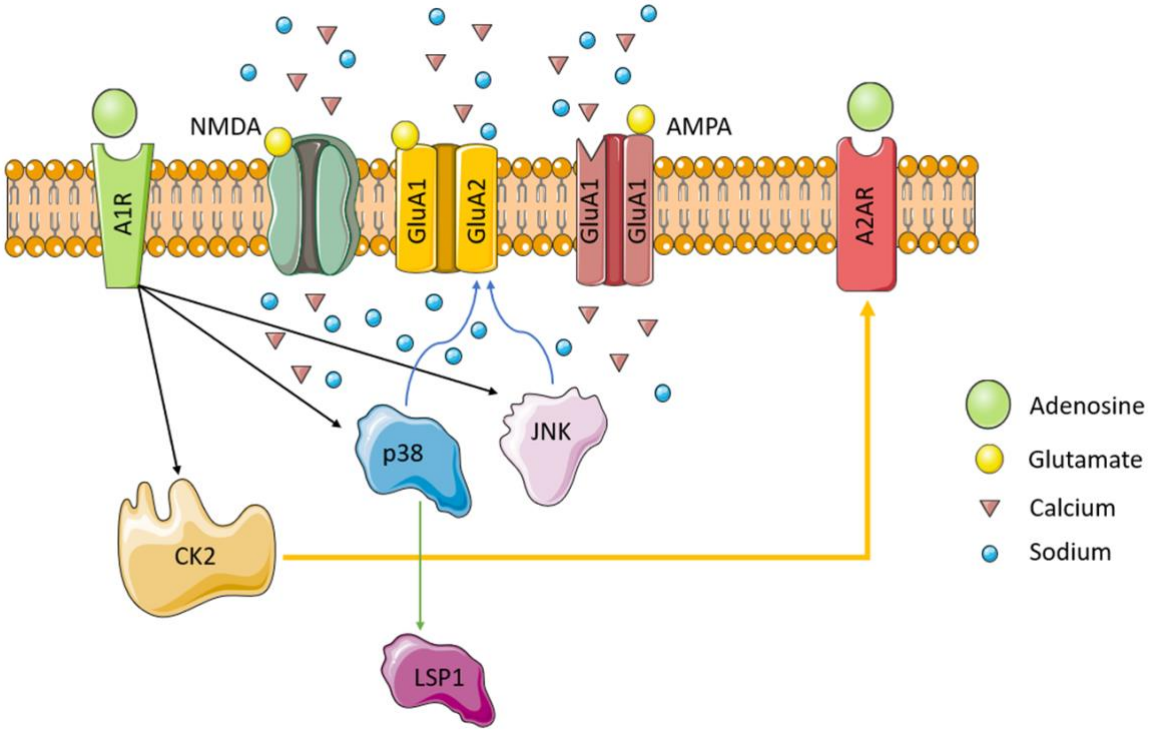
1.5 Adenosine signalling and LTP

Adenosine signalling is also involved in synaptic plasticity, which associates with learning and memory. The level of LTP indicates how strong the synaptic strength is. Memory loss is considered to associated with impairment of LTP. There are two kinds of LTP, decaying LTP and non-decaying LTP, Dong's lab suggested that restoration of AMPAR endocytosis can convert non-decaying LTP into decaying LTP (Dong et al. 2015). Since in the previous description, it has been demonstrated by our lab that adenosine A1 receptor is involved in AMPAR endocytosis. Moreover, both adenosine A1 and A2A receptors (A1Rs, A2ARs) have proved to be crucial to synaptic plasticity. A1R agonists such as CPA, attenuate LTP, whereas selective antagonists of A1Rs, such as DPCPX, facilitate LTP (De Mendonça and Ribeiro 2001). A potent A2AR agonist, 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS 21680), facilitated LTP in rat hippocampal slices (De Mendonça and Ribeiro 1994) while A2AR antagonist, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261), attenuated LTP (Almeida et al. 2003). These findings encourage us to further explore the relationship between adenosine signalling, AMPARs and LTP.

1.6 Adenosine Receptors and LSP1

Leukocyte-specific protein 1 (LSP1) is a small protein which is widely expressed in immune system, including monocytes, macrophages, neutrophils, and endothelium pre-B cells and B cells (Le et al. 2015). LSP1 serves an important downstream substrate of p38 mitogen-activated protein kinase (p38 MAPK) (Liu et al. 2005). In the Cayabyab Lab, we discovered that in rat hippocampal brain slices, A1R is associate with the activation of p38 MAPK, which further regulates GluA2-containing AMPAR endocytosis (Chen et al. 2014). These results provide a very intriguing clue regarding the possible novel role of LSP1 in A1R regulation on AMPARs and synaptic plasticity after stroke or hypoxia (see illustration below).

The following illustration depicts the major molecular players under investigation in this current study. In brief, we propose that during hypoxic insult to the brain, both of the major adenosine receptor subtypes in the hippocampus, namely the inhibitory A1Rs and stimulatory A2ARs, alter the expression levels and the properties of AMPARs (but not NMDARs) after the post-hypoxia reperfusion period. We suggest that novel molecular players in adenosinergic signalling, including the serine/threonine protein kinase CK2, the F-actin-binding protein LSP1, and the MAPKs (p38 and JNK), are important downstream targets of A1Rs during and after hypoxic insults. The current study provides a foundation for future investigations of these signalling pathways in hypoxic/ischemic brain damage.



2. Hypothesis and Objectives

Overall Hypothesis: We hypothesize that hypoxia induces A1R-mediated, dynamin-dependent internalization of A1Rs and GluA2/GluA1 AMPARs, which is then followed by an A2AR-mediated upregulation of these AMPARs, which is a prerequisite for full expression of APSP and increased neuronal damage.

Overall Objectives:

1. Using fEPSP recordings, I will determine the cellular and molecular basis for the adenosine-mediated post-hypoxia synaptic potentiation (APSP), in conjunction with known AMPAR antagonists.

2. I will determine whether there is a functional cross-talk between A1R and A2AR, and whether this contributes to neuronal damage after hypoxia.

3. I will also determine the novel role of the protein LSP1 in the brain, and specifically provide early evidence of whether this protein contributes to A1R-mediated changes in synaptic plasticity.

4. I will determine whether revisiting the potential for AMPAR antagonist as effective neuroprotective therapy with the advent of Perampanel, can provide the basis for further preclinical and clinical studies in stroke treatments.

3. Material and Methods

3.1 Animal Subjects

Animal care and all experimental procedures were performed by following the guidelines of the Canadian Council for Animal Care (CCAC) and were in line with the ARRIVE guidelines. Animal related experimental procedures are carried out under the supervision of Animal Care and Supply in the University of Saskatchewan Committee (Approved Animal Use Protocol Number: 20070090). Post-natal male Sprague-Dawley rats (20–30 days) were caged in groups of four. Housing temperature was maintained at 20 °C to 24 °C with a natural light (12 hours)/dark (12 hours) cycle. Rats were purchased by staff at the Lab Animal Service Unit (LASU) from Charles River Canada in Montreal. LSP1-deficient mice (knockout [KO]) (12-15 weeks) with wild type (WT) 129/SvJ background were generated and provided to Dr. Lixin Liu's lab (collaborator, Department of Pharmacology, University of Saskatchewan) as gift from the University of Toronto. Animal protocols applied on the control group (WT, 129/SvJ) in this study were approved by the Animal Care and Supply committee at the University of Saskatchewan. All rats and mice used in this study had unlimited access to tap water and standard pelleted diet provided by staff at LASU.

3.2 Preparation of Hippocampal Slices

Sprague-Dawley rats (postnatal 20-30 days), WT mice (12-15weeks), LSP1-deficient KO mice (12-15 weeks) were anaesthetized with halothane before utilization. All rats used are male.Brains are acquired after rapidly decapitated of the animals and were immediately transferred and submerged in ice-cold and oxygenated (aerating with 95% O_2 and 5% CO_2) The recipe for high concentration sucrose dissection solution is described as following (in mM): 87 NaCl, 7.0 $MgCl_2$,1.25 NaH_2PO_4, 1.25 NaH_2PO_4, 25 glucose, 25 $NaHCO_3$, 2.5 KCl and 0.5 $CaCl_2$ ($CaCl_2$ was added after the solution being oxygenated) (Brust et al. 2007; Chen et al. 2014). Fully automated vibrating blade microtome (VTS1200S, Leica Instruments, Germany) was used to obtain hippocampal slices at 400 μm thickness. Brain slicing were conducted in the same ice-cold and oxygenated high concentration sucrose solution described as above.

Hippocampal slices were incubated in the solution for more than 90 min before further experiments were performed at 20 °C to 24 °C in oxygenated artificial cerebrospinal fluid (aCSF). The recipe for aCSF is decribed as following (in mM): 2.5 KCl, 126 NaCl, 26 $NaHCO_3$, 2.0 $MgCl_2$, 10 glucose 1.25 NaH_2PO_4 and 2.0 $CaCl_2$ ($CaCl_2$ was added after the solution being oxygenated) (Brust et al. 2007; Chen et al. 2014).

3.3 Electrophysiology

Continuous perfusion of oxygenated aCSF at 3 ml/min with different drug treatment in the electrophysiology recording chamber was started before performing electrical stimulation of hippocampal slices. A bipolar tungsten electrode was used for stimulating the Schaffer collateral pathway to evoke field excitatory postsynaptic potentials (fEPSPs) by orthodromic stimulation. Recordings of the fEPSP signals were performed by a glass microelectrode filled with aCSF which was placed in CA1 *stratum radiatum*. A high-resolution, low-noise digitizer Digidata® 1440A was used to digitize the fEPSP signals. Signals were analyzed using Axon™ pCLAMP® 9 Electrophysiology Data Acquisition & Analysis Software (Foster City, CA). During every experiment, the fEPSPs were evoked by 0.1ms stimulation for every 30 seconds. Before choosing the standard stimulation amplitude for fEPSPs signals, a maximum amplitude was evoked for every hippocampal slice after a 20-min perfusion period of aCSF. For long term potentiation studies, the stimulation amplitudes were reduced to around one third of the maximum amplitude. For other electrophysiology studies in this project, the stimulation amplitudes were reduced to around half of the maximum amplitude. Stimulation voltage were mildly adjusted in case that the baseline is unstable. Baseline recording was conducted for at least 10 min to ensure a stable baseline fEPSP recording. Hypoxia treatment described in the following experiments was accomplished by aerating the aCSF with the mixture of 95% N_2 and 5% CO_2 for at least 20 minutes. Despite the fact that the recording chamber was open to air and the aerating solution was placed in an open container, oxygen in the solution was replaced by nitrogen, so the solution was considered hypoxic. For Chemically induced long-term potentiation (cLTP) experiments, cLTP was achieved by the treatment of 10 min Forskolin (50 μM) and Rolipram (0.1 μM) in a Mg^{2+}-free aCSF solution. After the induction of 10-min

cLTP, fEPSPs were recorded during a 60 minutes normoxic washout period in normal aCSF (Mg^{2+}-containing). A 10min baseline was recorded for the stableness of the signal. fEPSP slopes were normalized based on the first 10 sweeps (i.e. 5 min) after the perfusion of each treatment started.

3.4 Propidium Iodide Staining

To indicate cell death in the rat hippocampus, propidium iodide (PI) was selected as a fluorescent marker due to its ability of entering cells with damaged plasma membranes (Pugliese et al. 2009). This commonly applied method was also recently described by our lab (Stockwell et al. 2016). For prior incubation experiments, after generating the rat hippocampal slices, I incubated the slices in the oxygenized aCSF solution containing the drug treatment for 60 minutes. Slices were then transferred to hypoxic aCSF solution for 20 minutes, followed by a 3-hour oxygenized aCSF washout. For normal perfusion experiments, slices were treated the with drug perfused at certain time points, and then washout for 3 hours. In the third hour of the washout, Propidium iodide (PI, 5 μg/ml) was added to the oxygenized aCSF solution. After adding PI, slices were immediately covered with aluminum foil. From this time point, all related procedures were conducted in the dark to prevent photobleaching of PI. Slices were then transferred in 12-well plates, with maximum of three slices in each well. After rinsing with aCSF, 4% paraformaldehyde were added in each well to fix the slices overnight at 4°C. On the second day of the experiment, slices were washed in 1X PBS for 10 minutes and then repeated twice. Slices were then transferred and mounted on glass microscope slides (two slices on one slide) after adding Prolong Gold Antifade Reagent. Confocal microscope Zeiss LSM 700 (Carl Zeiss, Germany) were used to image the slices. PI fluorescence were induced by 543 laser (green). 10 X objective lens was used to image the whole hippocampal slice and Zeiss Plan-Apochromat 63 X /1.4 oil objective lens (Carl Zeiss, Germany) was used to image the CA1 region of the hippocampal slices. Background parameters were first determined by slices with the most observed fluorescence (treatment by hypoxia alone) and were applied in all other treatment groups. The images of the hippocampal CA1 region were captured using Z-stack. Each Z-stack image was captured at 2 μm with 200 μm depth into the slices. For densitometry

analysis, the average of two Z-stack image were captured for each slice. Zeiss Zen 2009 version 5.5 software (Carl Zeiss, Germany) were used to perform image capture. Image data were analyzed by ImageJ. The top and bottom Z-stack images were not included in the analyze since neuronal damage in these regions were partially caused by slicing procedure. The final images were generated in Adobe Photoshop CC (Adobe Systems, OS X operating system) by assembling the captured image into the whole hippocampal slice.

3.5 Biochemistry Studies

Rat hippocampal brain slices generated by previously described procedures (Chen et al. 2014, 2016; Stockwell et al. 2016) were washed three time in aCSF before transferred into 1 mg/ml NHS-SS-Biotin containing 4°C aCSF. After 45 minutes incubation, slices were then transferred into quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3) to stop the reactions, followed by 10 minutes washing in aCSF for three times. Slices were then homogenized in lysis buffer (1 mM EDTA, 1 mM NaF, 50 mM Tris, 150 mM NaCl, 10 μg/μl aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM Na_3VO_4, 20 mM sodium pyrophosphate, 1 mM PMSF) with 1% NP-40 and centrifuged to obtain the supernatant. Bradford Assay were performed to determine the protein concentrations. 500 μg of the biotinylated protein lysates in each treatment were diluted in lysis buffer. Equal amount of streptavidin beads was added to the lysates before incubation overnight at 4°C. On the following day, beads were washed with lysis buffer containing 0.1% NP-40 for 4 times. Each protein sample was then boiled at 95°C for 5 minutes with 50 μl of 2 X Laemmli sample buffer. Samples were separated by running through 10% polyacrylamide gel and transferred to PVDF membranes for 3 hours at 0.4 A. Membranes were then blocked in 5% nonfat milk in TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base) for one hour and probed with GluA1, GluA2 and GAPDH primary antibodies in TBST with 5% nonfat milk and 0.025% sodium azide at 4°C overnight. On the following day, membranes were washed four time in TBST (15 minutes each) and incubated with appropriate mouse or rabbit secondary antibodies (1:1000) for one hour. After washing in TBST for four times (15 minutes each). Enhanced chemiluminescence (ECL) reagent (Bio-Rad) were applied on the membrane

to visualizing the proteins. Both molecular weight of the biotinylated lysates and whole cell lysates are determined based on following information: GluA1 (100 kDa), GluA2 (100 kDa) and GAPDH (37 kDa).

3.6 Drug Treatments

N^6-cyclopentyladenosine (CPA) (Sigma) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma) were selected as A1R agonist at 5 mg/kg and A1R antagonist at 5 mg/kg, respectively. The amino acide sequence of the Tat-GluA2-3Y (YG) (GL Biochem) peptide is described as following YGRKKRRQRRR-^{869}YKEGYNVYG^{877} and the scrambled version: YGRKKRRQRRR-VYKYGGYNE. The function of YG and scrambled YG (GL Biochem) are described in previous studies. (Chen et al. 2015) CPA, DPCPX, YG peptide and scrambled YG were dissolved in DMSO (Sigma) before intraperitoneal injection (i.p. injection) were performed on animals. Bath applied drug in electrophysiology studies are describe as following: 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris), potent AMPAR antagonist; 6,7-Dinitroquinoxaline-2,3-dione (DNQX) (Tocris), non-NMDAR antagonist; 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Tocris), AMPAR antagonist; (5R,10S)-(-)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cylcohepten-5,10-imine maleate (MK 801) (Tocris), NMDAR antagonist; D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Tocris), NMDAR antagonist; N-[3-[[4-[(3-Aminopropyl)amino]butyl]amino]propyl]-1-naphthaleneacetamide trihydrochloride (NASPM) (Tocris), Ca^{2+}-permeable AMPAR antagonist; (S)-N-[7-[(4-Aminobutyl)amino]heptyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide dihydrochloride (Philanthotoxin 74) (Tocris), GluA1- and GluA3-containing AMPAR antagonist; and N,N,H,-Trimethyl-5-[(tricyclo[3.3.1.13,7]dec-1-ylmethyl)amino]-1-pentanaminiumbromide hydrobromide (IEM 1460) (Tocris), non-GluA2-containing AMPARs. All bath applied drugs were dissolved in DMSO before adding to aCSF. In each treatment, the final concentration of DMSO is less than 0.1%. Dynasore hydrate, a GTPase dynamin inhibitor, was purchased at Sigma, and also dissolved in DMSO before applied to bath treatment.

3.7 Statistical Analysis

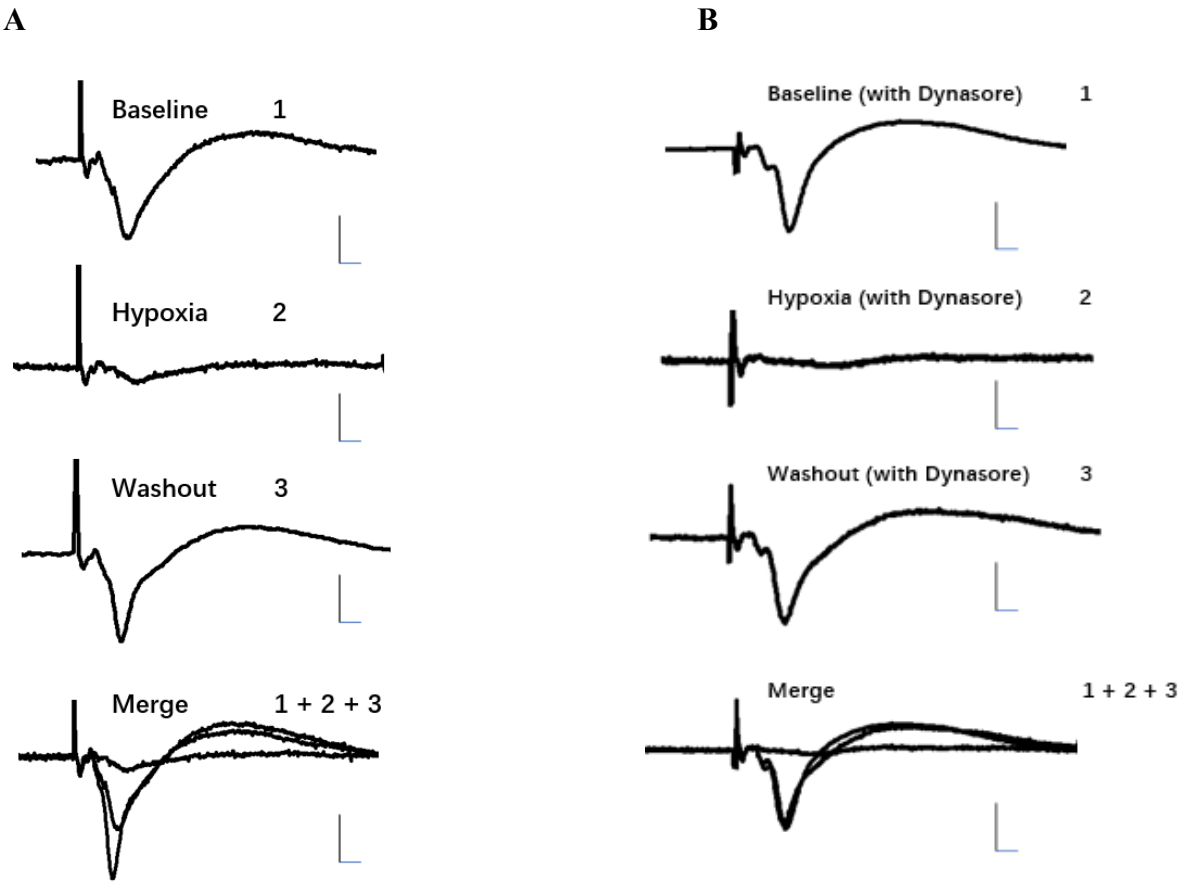
PI staining densitometry are calculated with Quantity 1, made by Bio-Rad and a public domain software, ImageJ. For biochemical analysis, a one-way ANOVA and post hoc Student-Newman-Keuls were performed in order to generate the significance of different treatment groups. For electrophysiology study analysis, Turkey-Kramer tests were performed on top of one-way ANOVA analysis. All statistical analysis was generated by GraphPad Prism InStat (Version 3.0) on the operating system of Windows 10.

4. Results

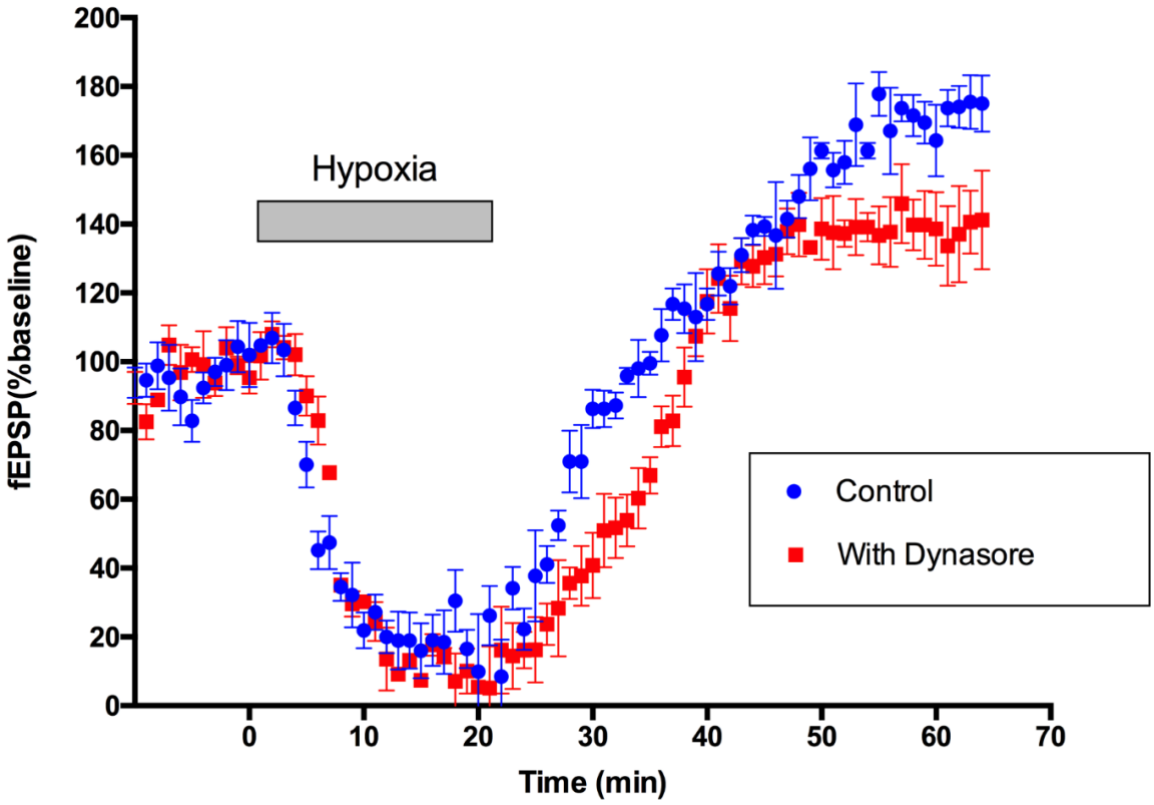
4.1 Full expression of APSP is regulated by clathrin-mediated, dynamin-dependent internalization of GluA1- and GluA2-containing AMPARs

Previously in our lab we have shown that prolonged A1R stimulation led to clathrin-mediated AMPA receptor endocytosis (Chen et al. 2014, 2016). We have also shown that during hypoxia, elevated extracellular adenosine in the hippocampus stimulates adenosine A1Rs and A2ARs and leads to adenosine-mediated post-hypoxia synaptic potentiation or APSP (Stockwell et al. 2016). Additionally, by using imaging techniques, we confirmed that in the hippocampal neurons, functional A1Rs are required for clathrin-mediated AMPAR endocytosis. However, A2ARs are not involved in the AMPAR endocytosis process. (Chen et al. 2014). Since the application of A1R antagonist DPCPX inhibits the level of APSP, it is crucial to test whether the inhibitory effect on APSP by this A1R antagonist requires the clathrin-mediated endocytosis pathway. Therefore, I first performed experiments comparing the level of APSP in absence or presence of Dynasore, a drug which has been used by our lab and other laboratories to block dynamin-dependent endocytosis (Chen et al. 2016; Macia et al. 2006). As shown in Figure 4.1, hippocampal slices perfused with 50 μM Dynasore showed attenuated level of APSP when compared to slice perfused with normal aCSF (Figure. 4.1 A-D). Next, I performed PI staining experiments to show that pre-incubation with Dynasore prevented hypoxia-induced cell death in rat hippocampal CA1 region (Figure. 4.1. E, F). Thus, these results suggest that prior stimulation of functional A1Rs and subsequent A1R internalization (i.e., dynamin-dependent) are required for the full expression of APSP, through clathrin-mediated AMPA receptor endocytosis pathway in rat hippocampal brain slices. These results also suggest that dynamin-dependent processes after hypoxia-induced adenosine receptor stimulation contribute to hippocampal neuronal damage.

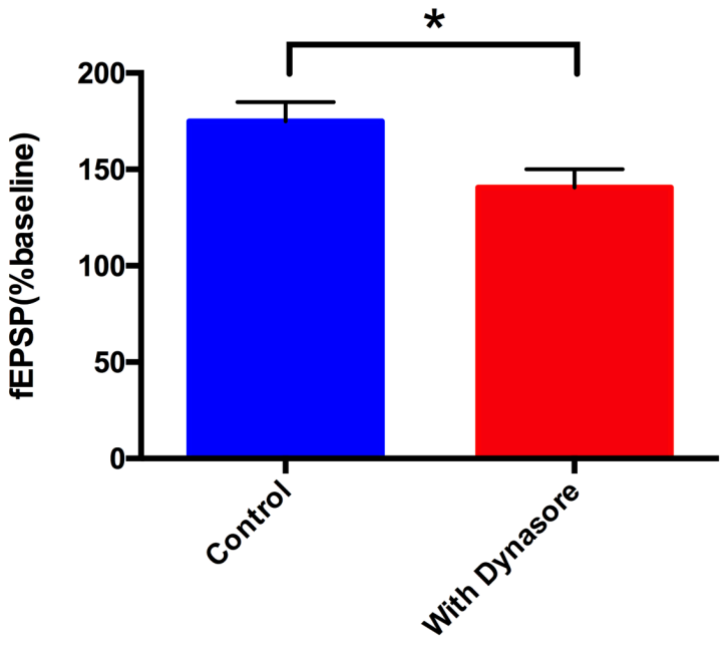
Figure 4.1.


A
Baseline 1
Hypoxia 2
Washout 3
Merge 1 + 2 + 3
B
Baseline (with Dynasore) 1
Hypoxia (with Dynasore) 2
Washout (with Dynasore) 3
Merge 1 + 2 + 3

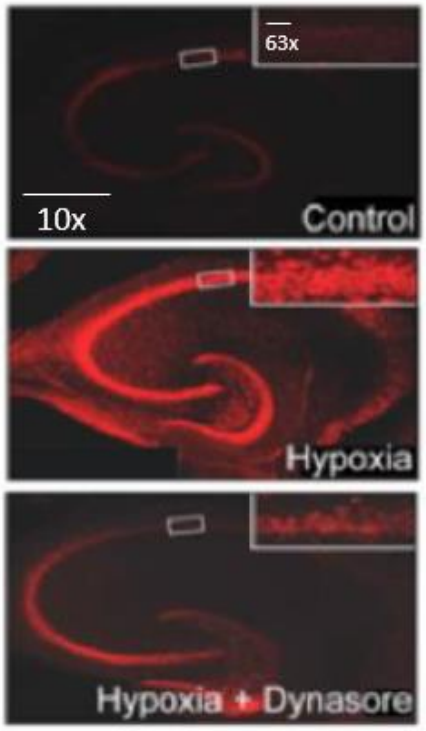
C


Hypoxia
fEPSP(%baseline)
Time (min)
Control
With Dynasore

D


*
fEPSP(%baseline)
Control
With Dynasore

E


63x
10x
Control
Hypoxia
Hypoxia + Dynasore

F

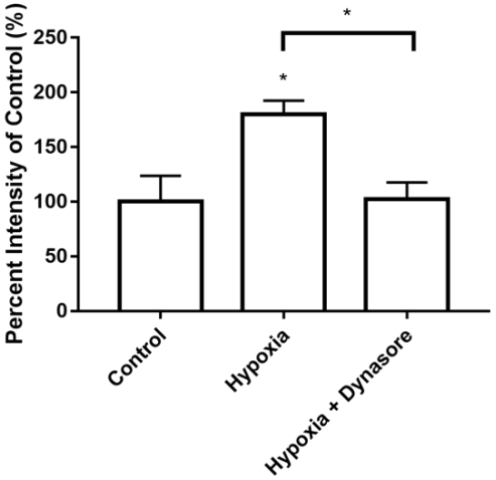
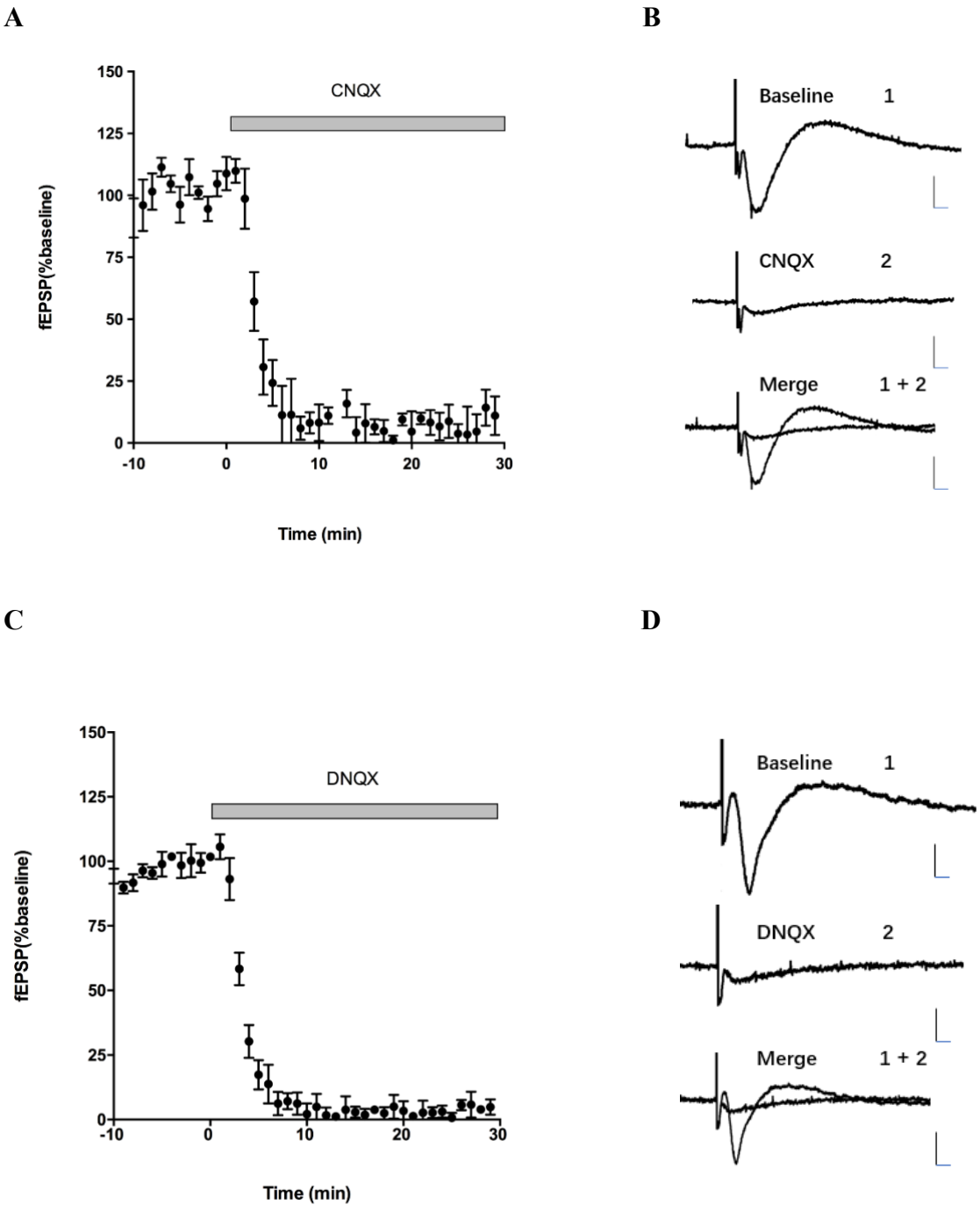

Percent Intensity of Control (%)
250
200
150
100
50
0
*
*
Control
Hypoxia
Hypoxia + Dynasore

Figure 4.1. Hypoxia (20 min) induced APSP and widespread neuronal death in acute hippocampal brain slices, as shown using propidium iodide (PI) staining. This neurodegeneration was attenuated by pre-incubation with the clathrin-mediated endocytosis inhibitor Dynasore. Slices pre-incubated with Dynasore also inhibited APSP levels after 20 min hypoxia treatment compared to control. A. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (1 + 2 + 3) showing the overlay of the three traces. B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (1 + 2 + 3) showing the overlay of the three traces. 50 μM Dynasore was perfused from the start of baseline. C. Summary time course plot showing mean fEPSP values with or without 50 μM Dynasore perfusion normalized to baseline (100%) D. Summary bar graph showing mean fEPSP values at the end of the normoxic washout period. Means ± SEM. Significance: * $p < 0.05$. N = 8 recordings per group. E. Representative images of hippocampal slices (large panels) stained with PI. Area CA1 (small panel, top right of each panel) was analyzed to compare relative PI intensity. Scale bars: 1mm (large panel) and 10 μm (small panel) apply to all confocal images F. Bar graph showing relative PI intensity in area CA1. Scale bars, 10 ms, 0.5 mV. Mean fluorescence intensities ± SEM are shown. Significance: * $p < 0.05$. N = 6 independent experiments.

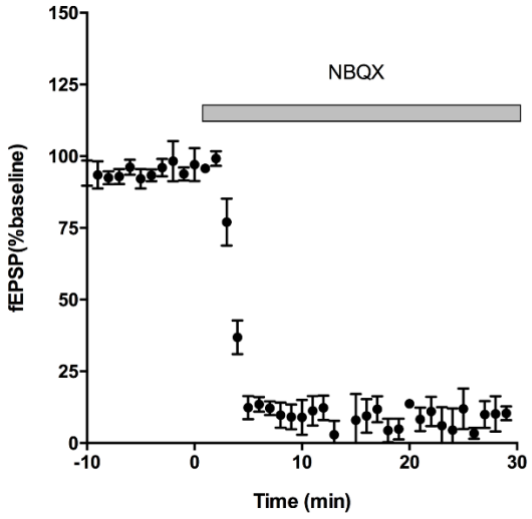
4.2 Synaptic transmission in rat hippocampal CA1 region is mainly mediated by AMPA-ergic mechanisms, instead of NMDA receptors

Previously, our lab showed that stimulation of adenosine A1Rs leads to GluA2 and GluA1 AMPAR internalization via clathrin-mediated and dynamin-dependent endocytosis (Chen et al. 2014). Thus, we hypothesized that AMPA-ergic receptors are mainly involved in mediating synaptic transmission in rat hippocampus. First, I perfused a potent AMAPAR antagonist, CNQX, which significantly decreased field EPSP levels in rat hippocampal brain slice recordings (Figure. 4.2-1 A, B). DNQX (Figure. 4.2-1 C, D), a selective non-NMDA receptor antagonist, showed the similar result. I also tested NBQX, a potent, selective and competitive AMPA receptor antagonist (Figure. 4.2-1 E, F), which had significant blocking effect on the EPSP. These AMPAR antagonists abolished around 95% of fEPSPs after 30 min of drug applications (Figure. 4.2-1 G). In previous studies, the excitotoxicity during the process of ischemic stroke is also shown to be mediated by NMDA receptors. Therefore, we also tested if NMDAR antagonists show similar effect as the AMPA-ergic receptor blockers. After applying D-AP5, a competitive NMDA antagonist (Figure.4.2-2 A, B), I did not observe any significant change in the amplitudes of field EPSP recordings, and similar results were shown with the application of MK-801, a potent non-competitive NMDA receptor antagonist (Figure. 4.2-2 C, D, summarized in Figure. 4.2-2 E). In fact, I observed a moderate (but non-significant) increase in synaptic transmission after MK-801 treatments. We concluded that the synaptic transmission in rat hippocampal CA1 region is mostly mediated by AMPA-ergic receptors, instead of NMDA receptors.

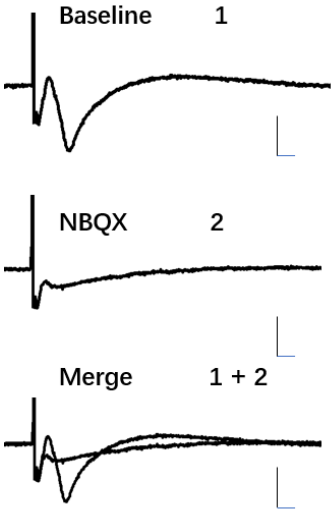
Figure 4.2-1.


A
B
C
D
CNQX
fEPSP(%baseline)
Time (min)
Baseline 1
CNQX 2
Merge 1 + 2
DNQX
Baseline 1
DNQX 2
Merge 1 + 2

E


NBQX
fEPSP(%baseline)
Time (min)
150
125
100
75
50
25
0
-10
0
10
20
30

F


Baseline 1
NBQX 2
Merge 1 + 2

G

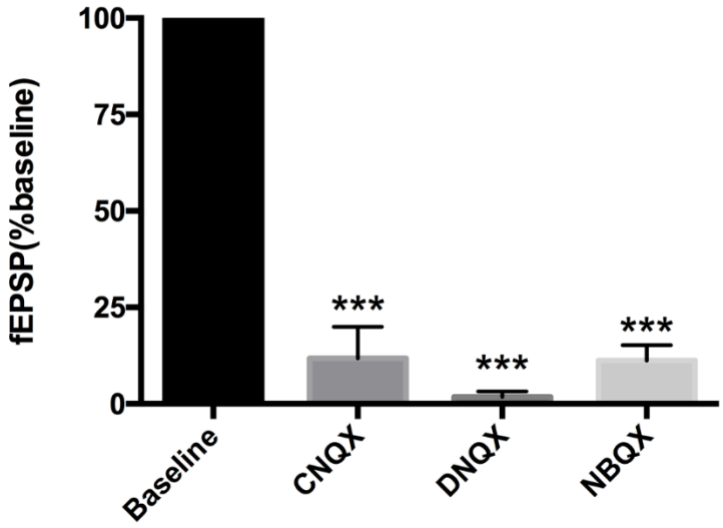
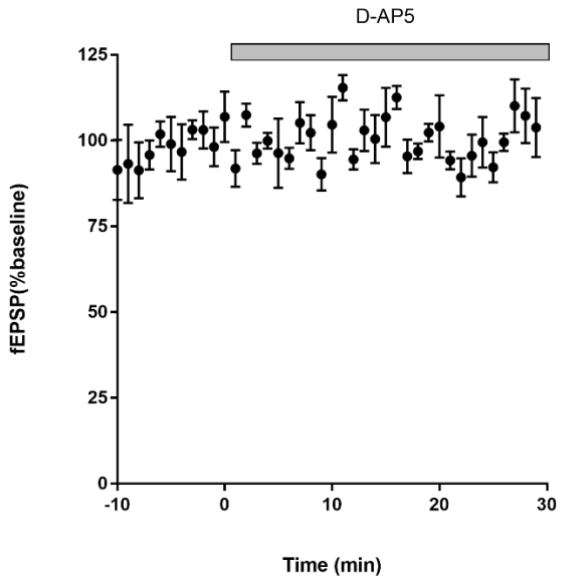

fEPSP(%baseline)
100
75
50
25
0
***
***
***
Baseline
CNQX
DNQX
NBQX

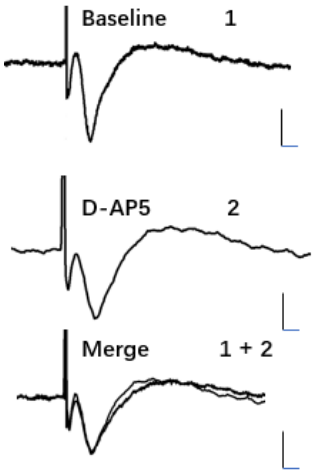
Figure 4.2-1. fEPSP levels were significantly blocked by AMPA-ergic antagonists CNQX, DNQX and NBQX. A. Summary time course plot showing mean fEPSP after application of CNQX (10 μM). B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 30 minutes CNQX treatment. (1 + 2) showing the overlay of the two traces. C. Summary time course plot showing mean fEPSP after application of DNQX (10 μM). D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 30 minutes DNQX treatment. (1 + 2) showing the overlay of the two traces. E. Summary time course plot showing mean fEPSP after application of NBQX (10 μM). F. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 30 minutes NBQX treatment. (1 + 2) showing the overlay of the two traces. G. Summary bar graph showing mean fEPSP values after application of CNQX (10 μM), DNQX (10 μM) and NBQX (10 μM). Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: *** $p < 0.005$. N = 7 recordings per group.

Figure 4.2-2.

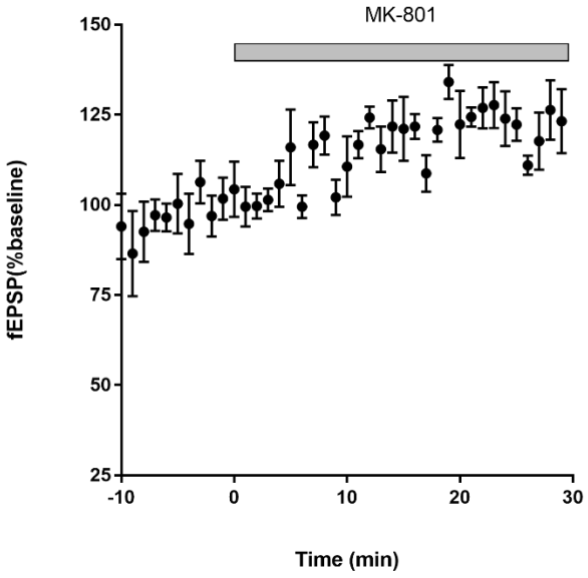
A



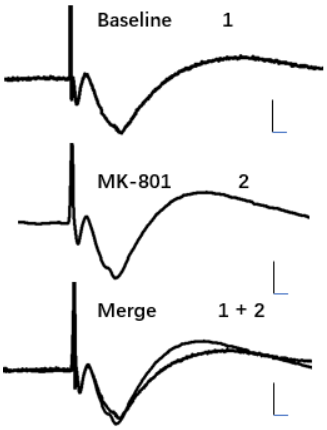
B



C



D



E

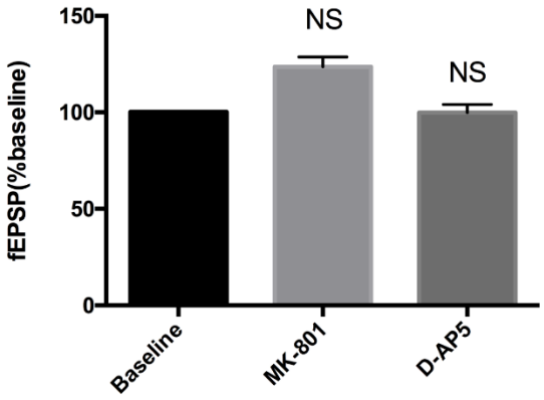
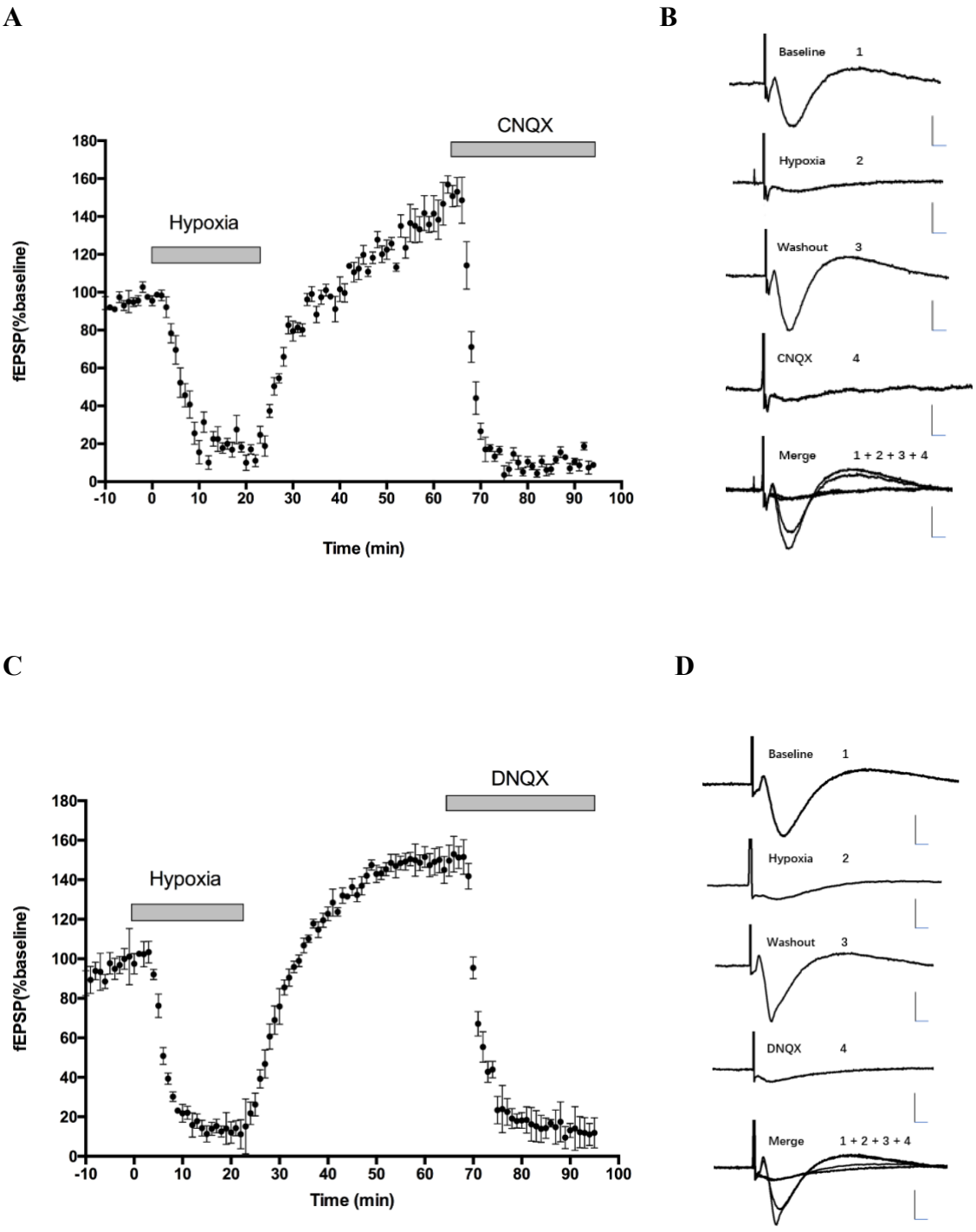


Figure 4.2-2. No significant changes were shown for fEPSP levels after applying NMDAR antagonists D-AP5 and MK-801. A. Summary time course plot showing mean fEPSP after application of D-AP5 (100 μM). B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 30 minutes D-AP5 treatment. (1 + 2) showing the overlay of the two traces. C. Summary time course plot showing mean fEPSP after application of MK-801 (5 μM). D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 30 minutes MK-801 treatment. (1 + 2) showing the overlay of the two traces. E. Summary bar graph showing mean fEPSP values after application of MK-801 (5 μM) and D-AP5 (100 μM). Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: NS $p > 0.05$. N = 7 recordings per group.

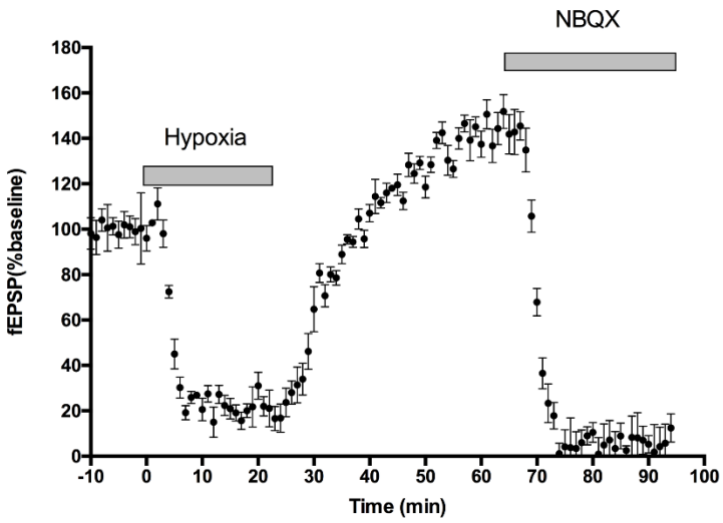
4.3 Adenosine induced post-hypoxia synaptic potentiation is also meditated by AMPA-ergic receptors instead of NMDA receptors.

We predicted that changes in synaptic transmission levels (i.e., enhanced fEPSPs) during the post-hypoxia period would be accompanied by increased neuronal susceptibility and may indicate delayed neuronal damage occurring after normoxic reperfusion of the rat hippocampal slices. In order to investigate which kind of glutamate receptors are regulating this process, I tested whether AMPA-ergic receptors are involved in the post-hypoxia synaptic potentiation that we normally observed during normoxic reperfusion. Surprisingly, the AMPAR antagonists CNQX, DNQX and NBQX abolished all the fEPSPs instead of merely decreasing the fEPSP levels back to baseline (Figure. 4.3-1 A-F, summarized in G). We initially hypothesized that the APSPs were mediated by enhanced NMDAR function. Therefore, we were also surprised that the tested NMDAR antagonists failed to show a similar effect as the AMPAR antagonists in abolishing the APSPs. Instead, when I perfused either MK-801 or D-AP5 following the 45 min reperfusion, I observed a moderate, but not significant, enhancement of fEPSPs in the rat hippocampal CA1 region (Figure. 4.3-2 A-D, summarized in E). Together, the results suggest that the APSP is mainly mediated by AMPAR-related mechanisms likely involving the A1R activation pathway.

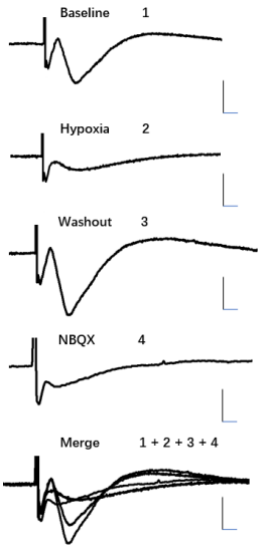
Figure 4.3-1.


A
B
C
D
Hypoxia
CNQX
DNQX
fEPSP(%baseline)
Time (min)
Baseline 1
Hypoxia 2
Washout 3
CNQX 4
DNQX 4
Merge 1 + 2 + 3 + 4

E


NBQX
Hypoxia
fEPSP(%baseline)
Time (min)

F


Baseline 1
Hypoxia 2
Washout 3
NBQX 4
Merge 1 + 2 + 3 + 4

G

-

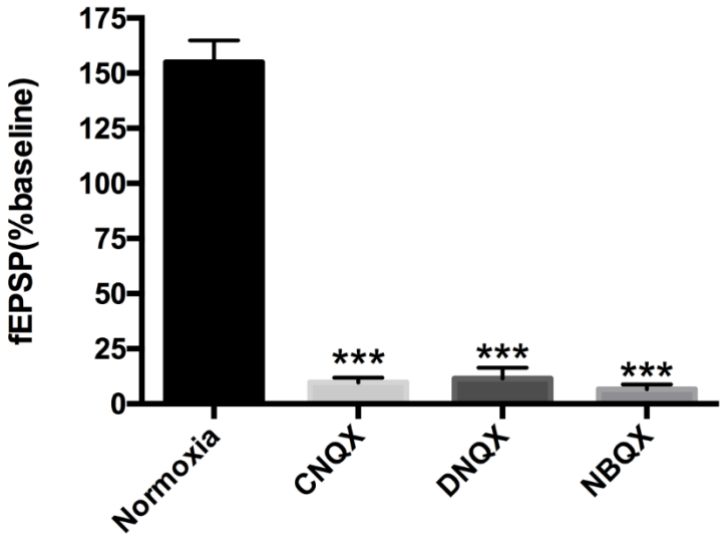
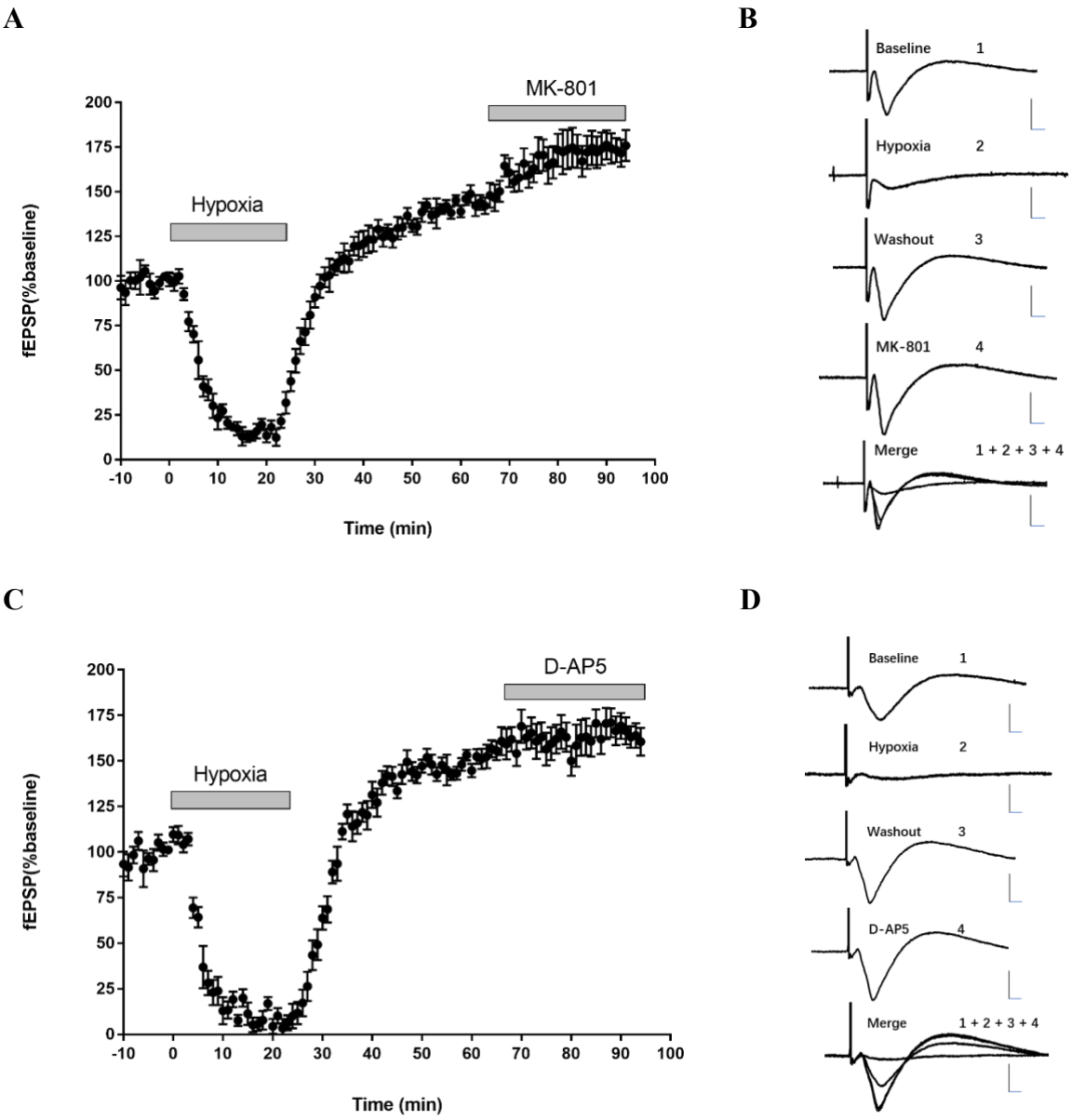

fEPSP(%baseline)
***
***
***
Normoxia
CNQX
DNQX
NBQX

Figure 4.3-1. fEPSP levels after post-hypoxia reperfusion were significantly blocked by AMPA-ergic antagonists CNQX, DNQX and NBQX. A. Summary time course plot showing mean fEPSP after application of CNQX (10 μM). B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes CNQX treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. C. Summary time course plot showing mean fEPSP after application of DNQX (10 μM). D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes DNQX treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. E. Summary time course plot showing mean fEPSP after application of NBQX (10 μM). F. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes NBQX treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. G. Summary bar graph showing mean fEPSP values after application of CNQX (10 μM), DNQX (10 μM) and NBQX (10 μM). Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: *** $p < 0.005$. N = 7 recordings per group.

Figure 4.3-2.


A
B
C
D
MK-801
Hypoxia
fEPSP(%baseline)
Time (min)
Baseline 1
Hypoxia 2
Washout 3
MK-801 4
Merge 1 + 2 + 3 + 4
D-AP5
D-AP5 4

E

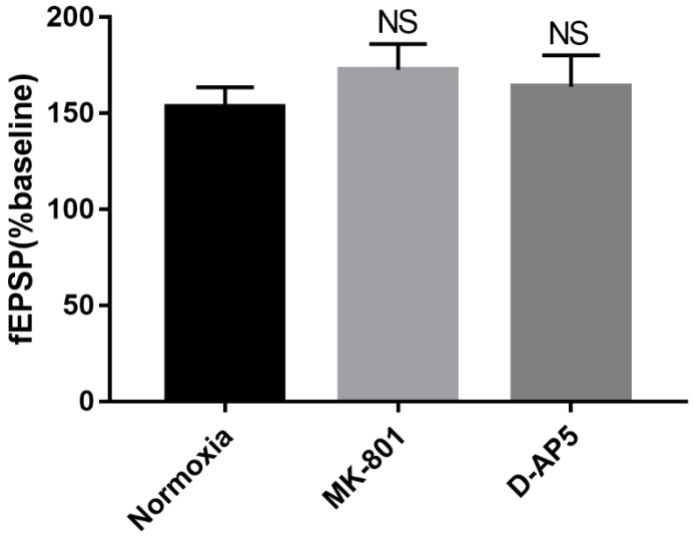


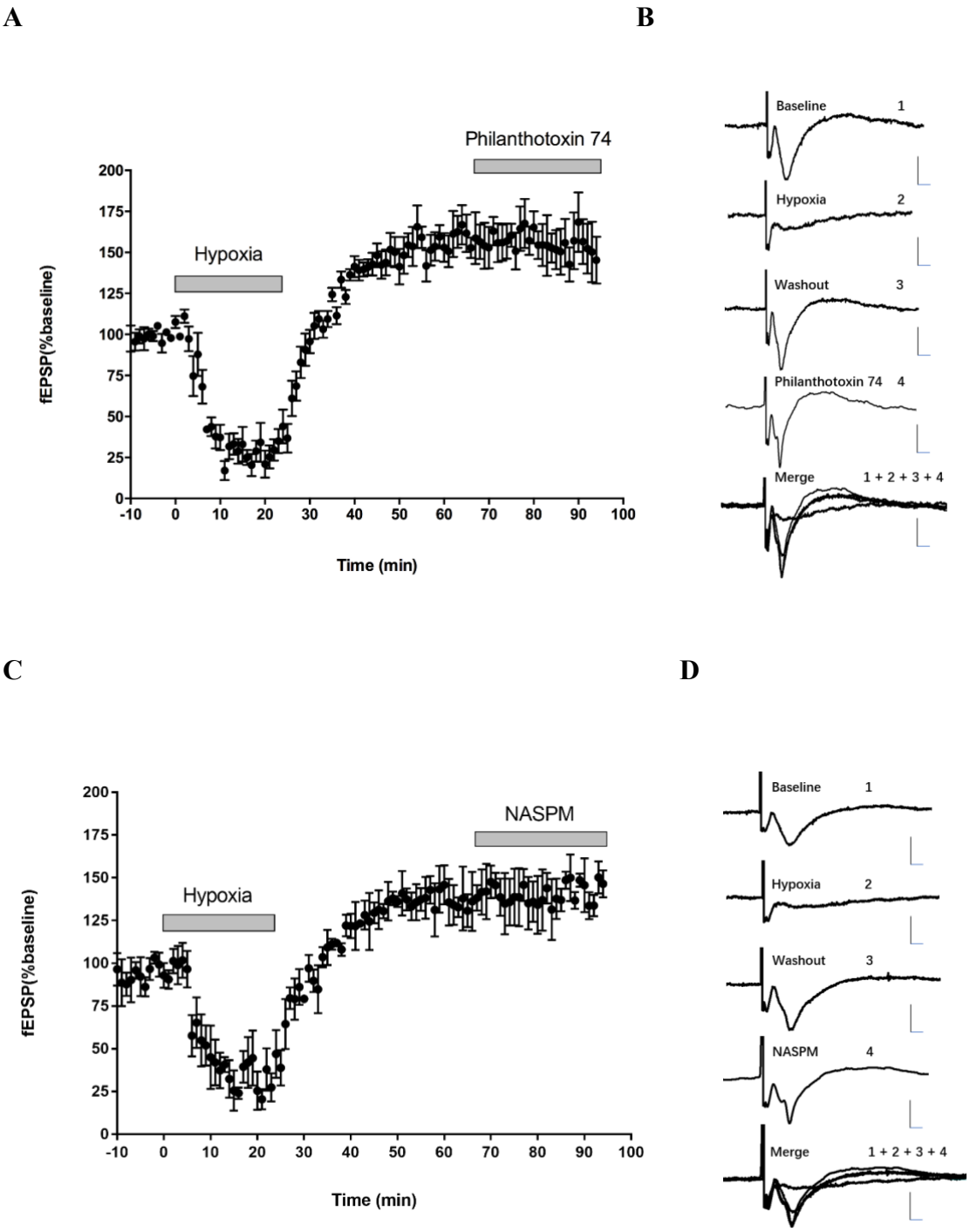
Figure 4.3-2. No significant change was observed for fEPSP levels during post-hypoxia period after applying NMDAR antagonists MK-801 and D-AP5. A. Summary time course plot showing mean fEPSP after application of MK-801 (5 μM). B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes MK-801 treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. C. Summary time course plot showing mean fEPSP after application of D-AP5 (100 μM). D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes D-AP5 treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. E. Summary bar graph showing mean fEPSP values after application of MK-801 (5 μM) and D-AP5 (100 μM). Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: NS $p > 0.05$. N = 7 recordings per group.

4.4 Ca^{2+}-permeable AMPAR blockers inhibited post-hypoxia synaptic transmission when applied early during hypoxia insult, but had no significant effect when applied after post-hypoxia reperfusion

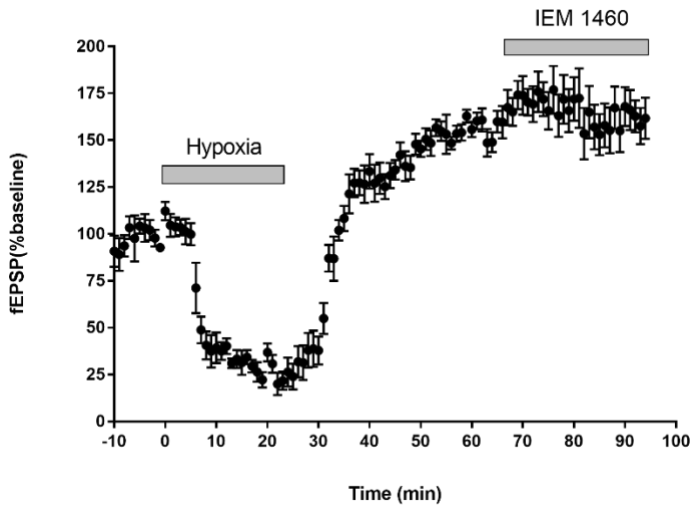
Since the post-hypoxia synaptic transmission was mostly mediated by an AMPA-ergic mechanism, I decided to test which type of AMPARs are involved in contributing to the APSP. I perfused Philanthotoxin-74, a selective inhibitor of Ca^{2+}-permeable AMPARs after APSP during CA1 recordings. Surprisingly, this antagonist did not modify the fEPSPs when applied 45 min after initiating the normoxia reperfusion (Figure. 4.4-1 A, B). Similar results were observed with either NASPM (Figure. 4.4-1 C, D), another Ca^{2+}-permeable AMPAR antagonist, or IEM 1460 (Figure. 4.4-1 E, F), a more selective antagonist for GluA2 subunit-lacking receptors over GluA2-containing receptors. Previous reports (Chen et al. 2014; Stockwell et al. 2016) suggested that chronic A1R stimulation leads to desensitization of A1Rs, which is then followed by increased insertion of A2ARs during ischemic conditions. It was also suggested that during hypoxic periods, both GluA1 and GluA2 surface levels remained depressed but that the GluA1 levels quickly recover upon normoxic reperfusion while GluA2 remained depressed (Stockwell et al. 2016). This led to the hypothesis that the A2AR stimulation by endogenous adenosine during normoxic reperfusion could induce rapid and transient insertion of calcium-permeable GluA1-containing AMPARs. Unpublished observations from our lab confirmed that pre-incubation with either DPCPX or SCH 58621 (A1R and A2AR antagonist, respectively) prevented the appearance of APSPs, which suggests a strong cross-talk between A1Rs and A2ARs. Therefore, we hypothesized that a transient insertion of GluA1-containing AMPARs during the early phase of normoxic reperfusion could contribute to the adenosine receptor dependent post-hypoxia synaptic potentiation. Thus, I tested whether early application of these same calcium-permeable AMPAR antagonists will show an inhibitory effect on the APSP levels. When drugs were applied after 5 min of hypoxia treatment until the end of the washout period, I did observe a decreased level of APSP after 45 min of reperfusion. (Figure. 4.4-2 A-D, summarized in E). These results suggested that delayed administration of Ca^{2+}-permeable AMPA receptor blockers have little neuroprotective effect on rat hippocampal slices whereas

application around the time of insult could attenuate the function of GluA1-containing AMPARs and decrease APSP levels and neuronal damage.

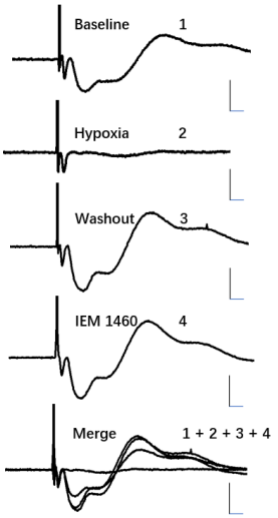
Figure 4.4-1.


A
B
Philanthotoxin 74
Hypoxia
fEPSP(%baseline)
Time (min)
Baseline 1
Hypoxia 2
Washout 3
Philanthotoxin 74 4
Merge 1 + 2 + 3 + 4
C
D
NASPM
Hypoxia
fEPSP(%baseline)
Time (min)
Baseline 1
Hypoxia 2
Washout 3
NASPM 4
Merge 1 + 2 + 3 + 4

E


IEM 1460
Hypoxia
fEPSP(%baseline)
200
175
150
125
100
75
50
25
0
-10
0
10
20
30
40
50
60
70
80
90
100
Time (min)

F


Baseline
1
Hypoxia
2
Washout
3
IEM 1460
4
Merge
1 + 2 + 3 + 4

G

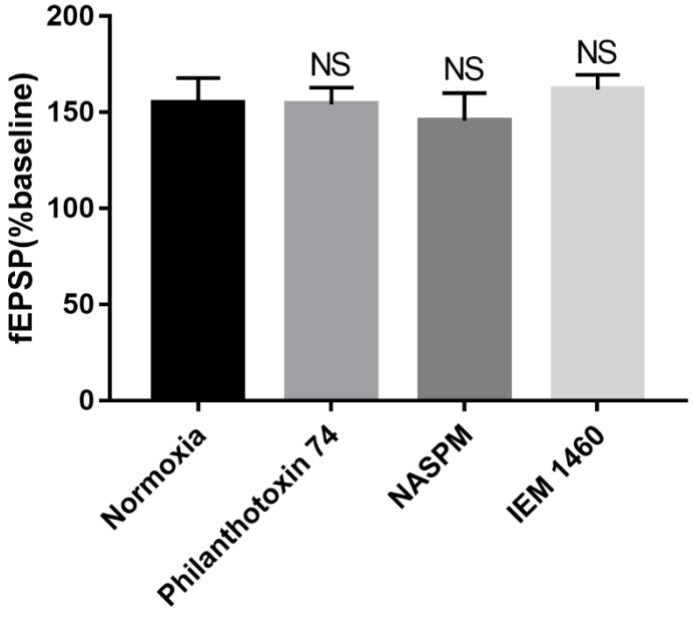
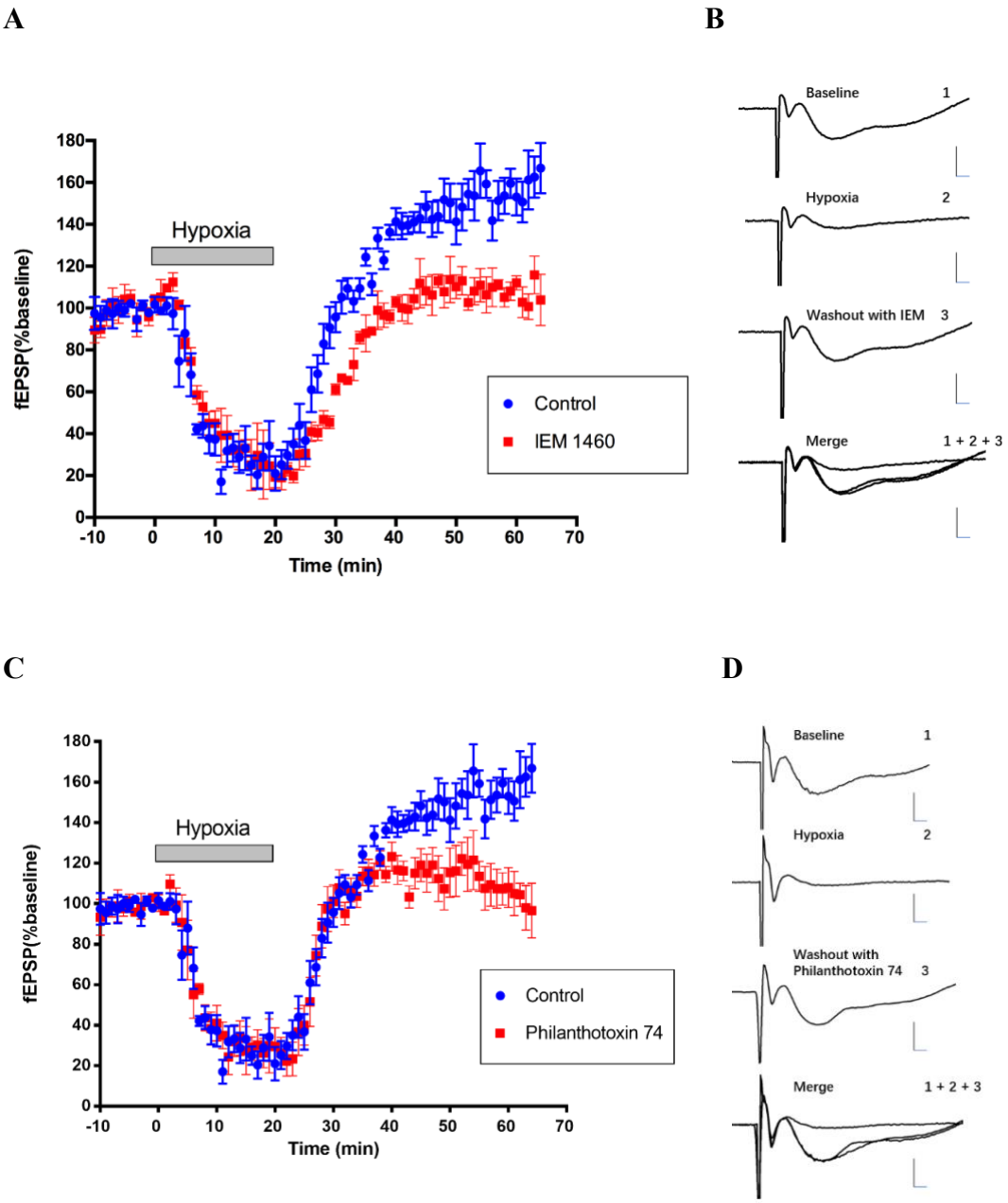

fEPSP(%baseline)
200
150
100
50
0
NS
NS
NS
Normoxia
Philanthotoxin 74
NASPM
IEM 1460

Figure 4.4-1. No significant change was observed in fEPSPs when Ca^{2+}-permeable AMPAR antagonists were applied 45 min after initiating the normoxia reperfusion. A. Summary time course plot showing the effect of Philanthotoxin-74 (50 μM) on fEPSPs when applied after 45 min of reperfusion. B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes Philanthotoxin-74 treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. C. Summary time course plot showing mean fEPSPs and the effect of applying NASPM (50 μM) 45 min after reperfusion. D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes NASPM treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. E. Summary time course plot showing mean fEPSPs and the effect of IEM 1460 (50 μM) after 45 min of reperfusion. F. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes IEM 1460 treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. G. Bar graph showing the lack of effect of Philanthotoxin-74 (50 μM), NASPM (50 μM) and IEM 1460 (50 μM) on APSPs when these drugs were applied 45 min after initiating normoxic reperfusion. Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: NS $p > 0.05$. N = 7 recordings per group.

Figure 4.4-2.


A
B
C
D
Hypoxia
fEPSP(%baseline)
Time (min)
Control
IEM 1460
Philanthotoxin 74
Baseline
Hypoxia
Washout with IEM
Washout with Philanthotoxin 74
Merge
1
2
3
1 + 2 + 3

E

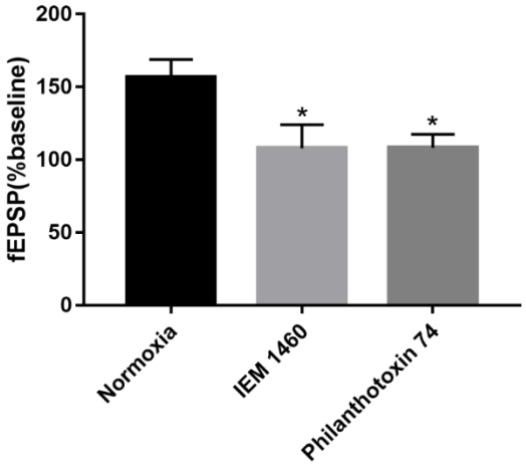

200
150
100
50
0
fEPSP(%baseline)
*
*
Normoxia
IEM 1460
Philanthotoxin 74

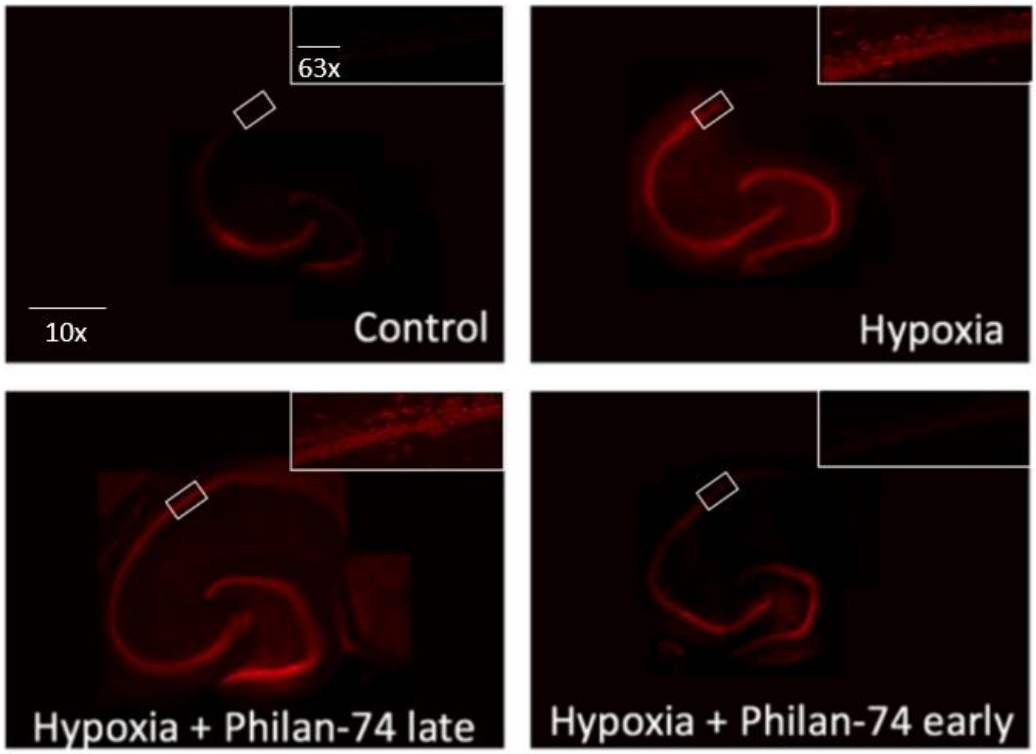
Figure 4.4-2. fEPSP levels after post-hypoxia reperfusion were attenuated when Ca^{2+}-permeable AMPAR inhibitors were applied after 5 min of hypoxia treatment. A. Summary time course plot showing mean fEPSPs and the effect of applying IEM 1460 (50 μM) soon after hypoxia treatment (i.e., 5min of start of hypoxia) and throughout normoxic reperfusion washout period. B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (1 + 2 + 3) showing the overlay of the three traces. IEM 1460 treatment was started from 5 min of hypoxia till the end of washout. C. Summary time course plot showing similar effect of Philanthotoxin-74 (50 μM) as IEM 1460 in inhibiting the APSPs when applied immediately after hypoxia treatment (i.e., 5 min of hypoxia onset) and throughout the normoxia reperfusion washout period. D. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (1 + 2 + 3) showing the overlay of the three traces. Philanthotoxin-74 treatment was started from 5 min of hypoxia till the end of washout. E. Summary bar graph showing mean fEPSP values of APSP and the inhibitory effects of IEM 1460 (50 μM) and Philanthotoxin-74 (50 μM) when applied 5 min after onset of hypoxia and lasting throughout the normoxia reperfusion washout period. Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: * $p < 0.05$. N = 7 recordings per group.

4.5 Early blockade of Ca^{2+}-permeable AMPARs prevents neuronal damage induced by hypoxia in rat hippocampus

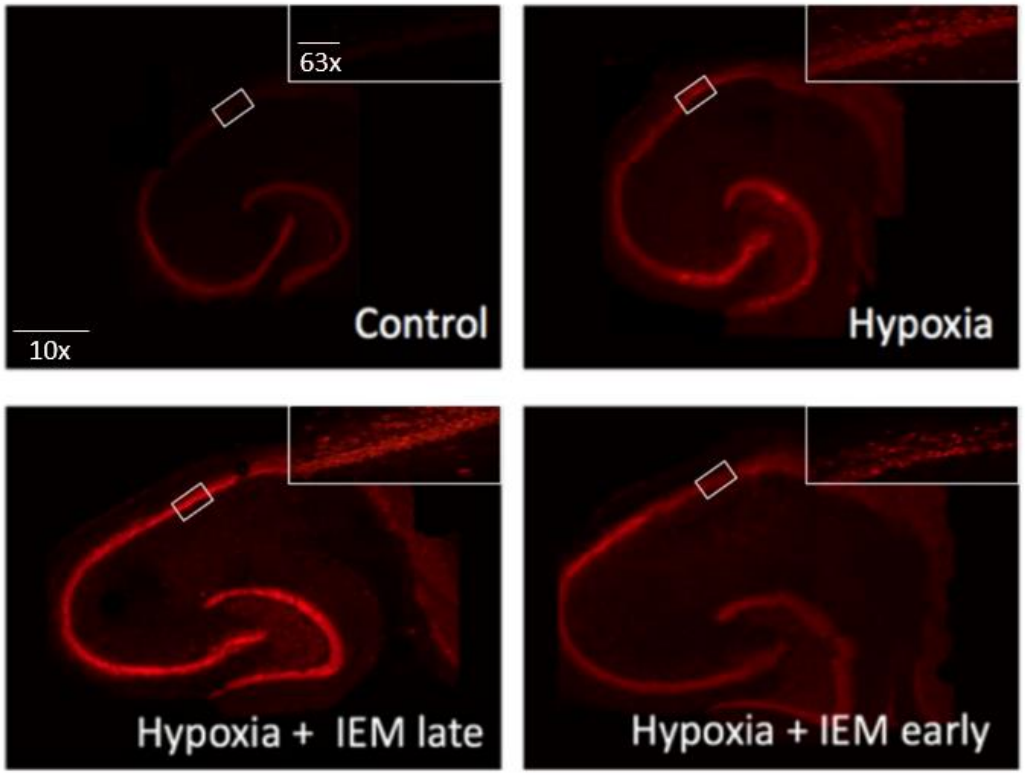
To further confirm my results on Ca^{2+}-permeable AMPAR blockers from above electrophysiological experiments, I performed propidium iodide (PI) staining experiments to compare levels of cell damage when Ca^{2+}-permeable AMPAR blockers were applied at different time points. Early application (5 min into hypoxia till the end of washout) of IEM 1460, Philanthotoxin-74 and NASPM in rat hippocampal slices both dramatically reduced cell damage compared to much later application of the drugs (i.e., 45 min of reperfusion) which produced no protection of hippocampal neurons (Figure. 4.5 A-C, summarized in D). These results showed that the adenosine-induced post-hypoxia synaptic potentiation (APSP) accompanying neuronal damage that we observed was likely due, in part, to increased function and transient insertion of CP-AMPARs. This novel mechanism may provide further insight into the possible reasons for failure of glutamate receptor blockers in clinical trials involving patients suffering from stroke.

Figure 4.5.

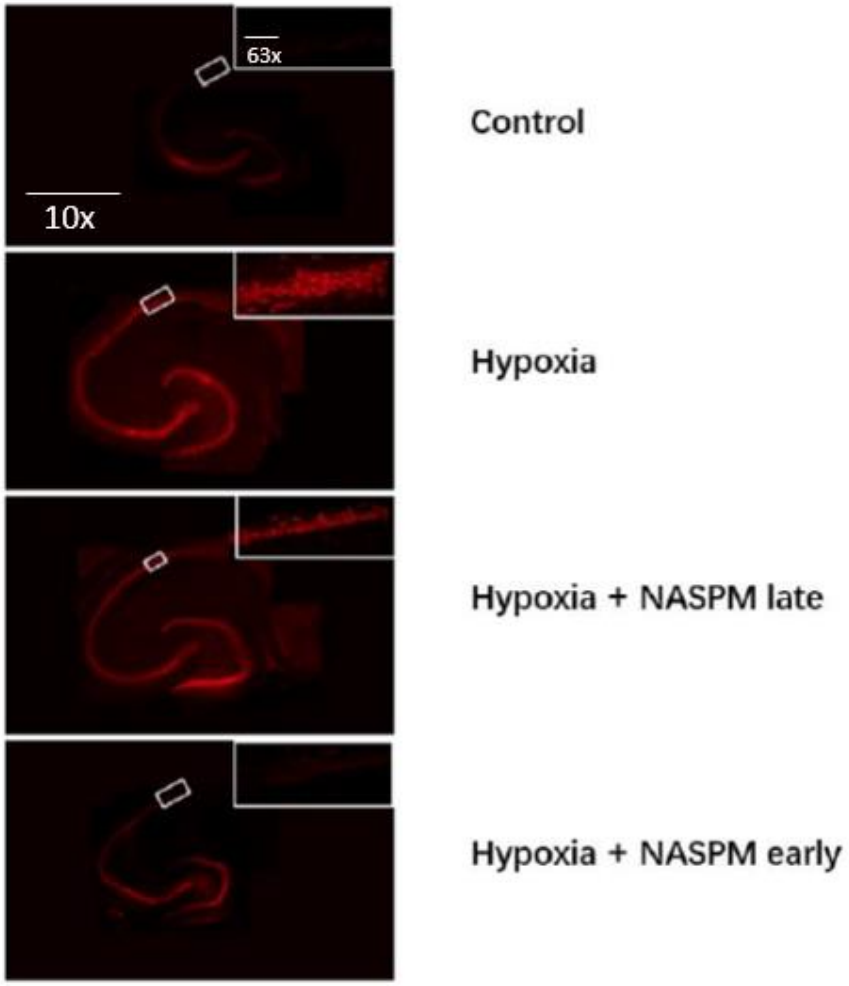
A


63x
10x
Control
Hypoxia
Hypoxia + Philan-74 late
Hypoxia + Philan-74 early

B


63x
10x
Control
Hypoxia
Hypoxia + IEM late
Hypoxia + IEM early

C


63x
10x
Control
Hypoxia
Hypoxia + NASPM late
Hypoxia + NASPM early

D

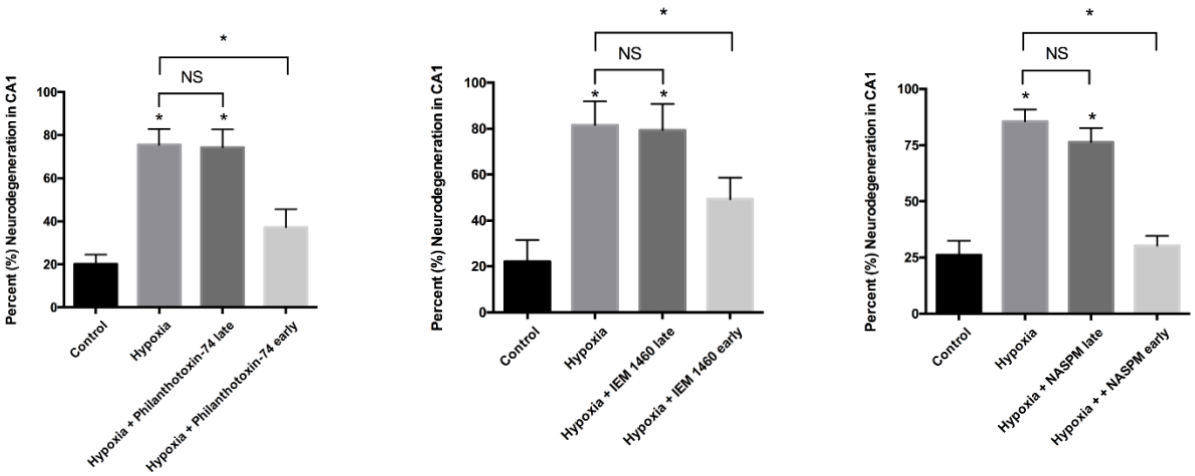
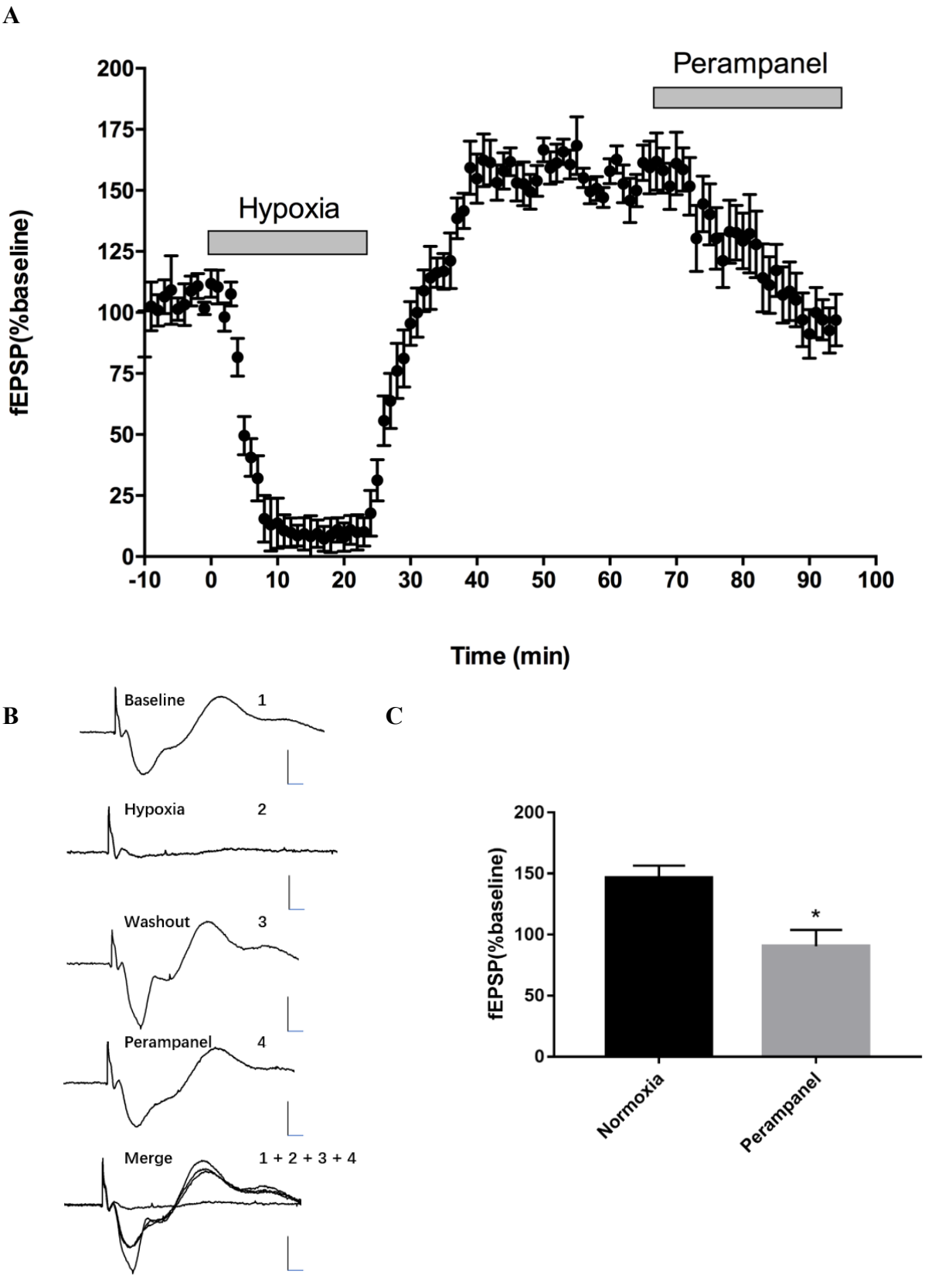

Percent (%) Neurodegeneration in CA1
NS
Control
Hypoxia
Hypoxia + Philanthotoxin-74 late
Hypoxia + Philanthotoxin-74 early
Hypoxia + IEM 1460 late
Hypoxia + IEM 1460 early
Hypoxia + + NASPM early

Figure 4.5. Hypoxia (20 min) induced widespread neuronal death in acute hippocampal slices, as shown using propidium iodide (PI) staining. This neuronal death was prevented with early application of Philanthotoxin-74 (50 μM), IEM 1460 (50 μM) or NASPM (50 μM) starting at 5 min into the hypoxic insult (early), but not with application of the blockers after the washout (late, 45 min after start of normoxic washout). A. Representative images of hippocampal slices (large panels) treated with hypoxia, hypoxia with early application of Philanthitoxin-74 (50 μM) and hypoxia with late application of Philanthitoxin-74, treated with PI. Area CA1 (small panel, top right of each panel) was analyzed to compare relative PI intensity. B. Representative images of hippocampal slices (large panels) treated with hypoxia, hypoxia with early application of IEM 1460 (50 μM) and hypoxia with late application of IEM 1460, treated with PI. Area CA1 (small panel, top right of each panel) was analyzed to compare relative PI intensity. C. Representative images of hippocampal slices (large panels) treated with hypoxia, hypoxia with early application of NASPM (50 μM) and hypoxia with late application of NASPM, treated with PI. Area CA1 (small panel, top right of each panel) was analyzed to compare relative PI intensity. D. Bar graphs showing relative PI intensity in area CA1. Mean fluorescence intensities ± SEM are shown. Significance: * $p < 0.05$. N = 6 independent experiments. Scale bars: 1mm (large panel) and 10 μm (small panel) apply to all confocal images.

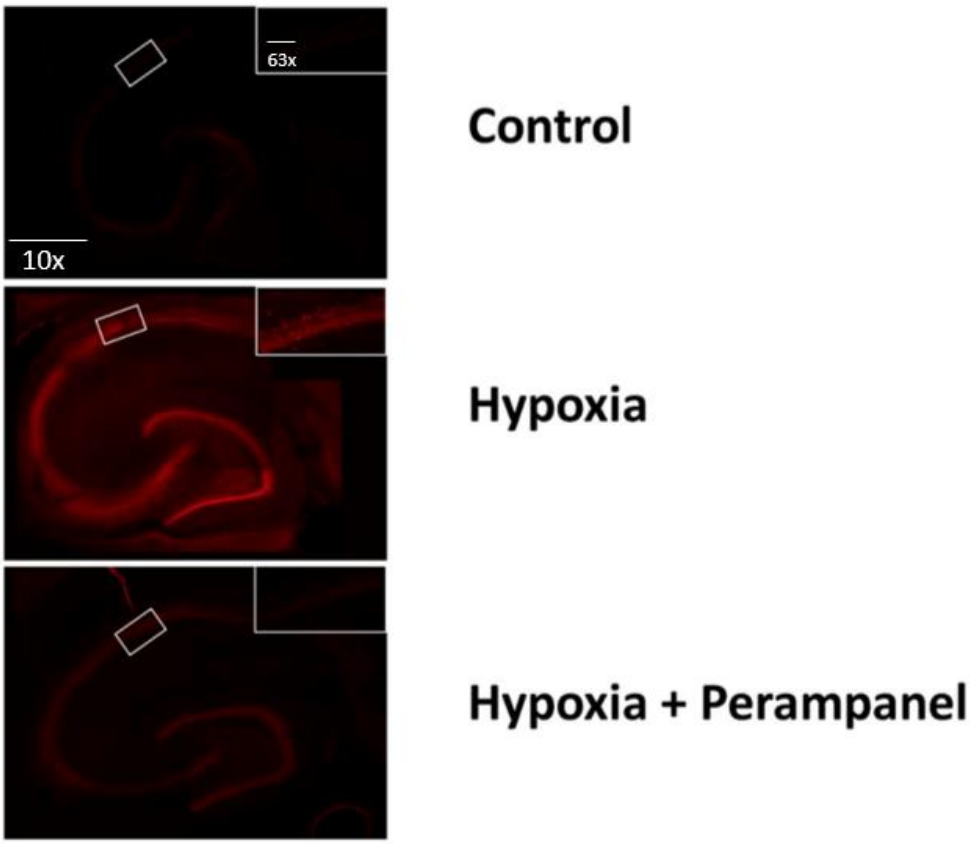
4.6 Perampanel attenuated post-hypoxia synaptic transmission and reduced neuronal damage

Since Perampanel is also a non-competitive AMPAR antagonist and it is currently clinically approved for so called "drug-resistant" epileptic patients (age≥12 years old), it is intriguing for me to investigate whether Perampanel has neuroprotective effects on rat hippocampal slices after hypoxic insult. Surprisingly, unlike the other CP-AMPAR blockers I previously tested, administration of Perampanel (200 nM) after the 45 min normoxic washout did not abolish the baseline synaptic transmission but did attenuate the level of APSPs back to baseline (Figure 4.6 A-B, summarized in C). These results suggested that Perampanel might represent a promising therapeutic agent to combat the delayed damaging effects of ischemia-reperfusion injury in stroke patients. However, the difference in the mechanism of action between Perampanel and other competitive AMPAR blockers still needs to be specified through further experiments. It is likely that Perampanel, in addition to having inhibitory effects on AMPARs, may have other off-target effects, such as a possible allosteric interaction with the $GABA_A$ receptor ($GABA_AR$) and thereby increasing the $GABA_AR$-mediated synaptic transmission in the hippocampus. The combined inhibitory effects on AMPAR/kainate receptor and stimulatory effects on $GABA_A$ receptor-mediated currents by the action of Perampanel may underlie the reduction in APSP during normoxic reperfusion (Figure 4.6 C) and reduction in hippocampal neuronal damage (Figure. 4.6 D, E), which is a mechanism that was suggested for the possible allosteric interaction of CNQX and subsequent increase in inhibitory synaptic transmission in the cerebellum (Brickley et al. 2001). Although this potentially novel dual effects of Perampanel on AMPARs (inhibitory) and $GABA_ARs$ (stimulatory) could be important to explain both the reduced levels of APSPs and neuronal damage during post-hypoxia normoxic washout, this is beyond the scope of my current studies but warrants further investigation in the future.

Figure 4.6.


A
Perampanel
Hypoxia
fEPSP(%baseline)
200
175
150
125
100
75
50
25
0
-10
0
10
20
30
40
50
60
70
80
90
100
Time (min)
B
Baseline
1
Hypoxia
2
Washout
3
Perampanel
4
Merge
1 + 2 + 3 + 4
C
fEPSP(%baseline)
200
150
100
50
0
*
Normoxia
Perampanel

D


63x
10x
Control
Hypoxia
Hypoxia + Perampanel

E

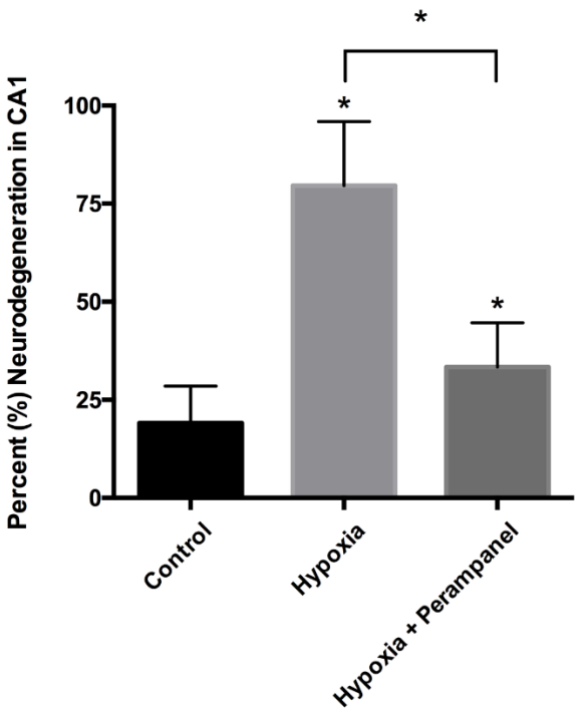
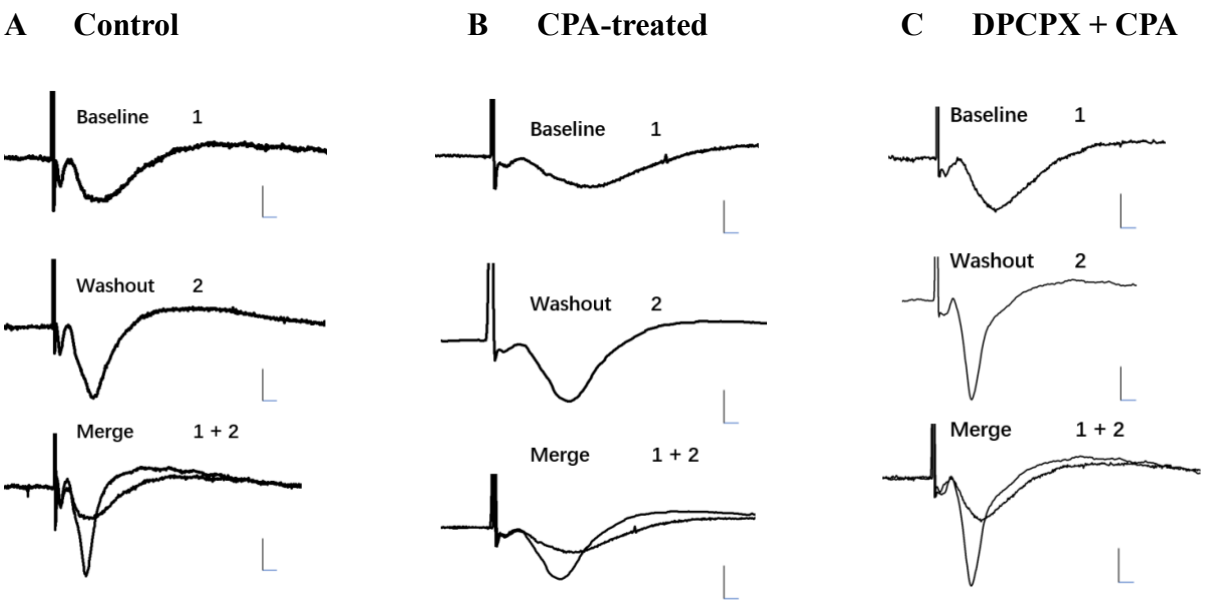

Percent (%) Neurodegeneration in CA1
100
75
50
25
0
*
*
*
Control
Hypoxia
Hypoxia + Perampanel

Figure 4.6. The fEPSP levels after post-hypoxia reperfusion were attenuated by Perampanel when applied after 45 min of reperfusion and Perampanel reduced neuronal death after post-hypoxia reperfusion. A. Summary time course plot showing the inhibitory effects of Perampanel (200 nM) on APSPs when applied 45 min after reperfusion. B. Sample fEPSP traces. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 20 minutes hypoxia treatment. (3) showing the average trace of the last 5 minutes of 45 minutes washout period. (4) showing the average trace of the last 5 minutes of the 30 minutes Perampanel treatment. (1 + 2 + 3 + 4) showing the overlay of the four traces. C. Summary bar graph showing mean fEPSP values of APSP after application of Perampanel (200 nM). Means ± SEM. Significance: * $p < 0.05$. N = 8 recordings per group. D. Representative images of hippocampal slices (large panels) treated with hypoxia, and hypoxia with application of Perampanel (1 μM), treated with PI. Area CA1 (small panel, top right of each panel) was analyzed to compare relative PI intensity. D. Bar graphs showing relative PI intensity in area CA1. Scale bars, 10 ms, 0.5 mV. Mean fluorescence intensities ± SEM are shown. Significance: * $p < 0.05$. N = 4 independent experiments. Scale bars: 1mm (large panel) and 10 μm (small panel) apply to all confocal images.

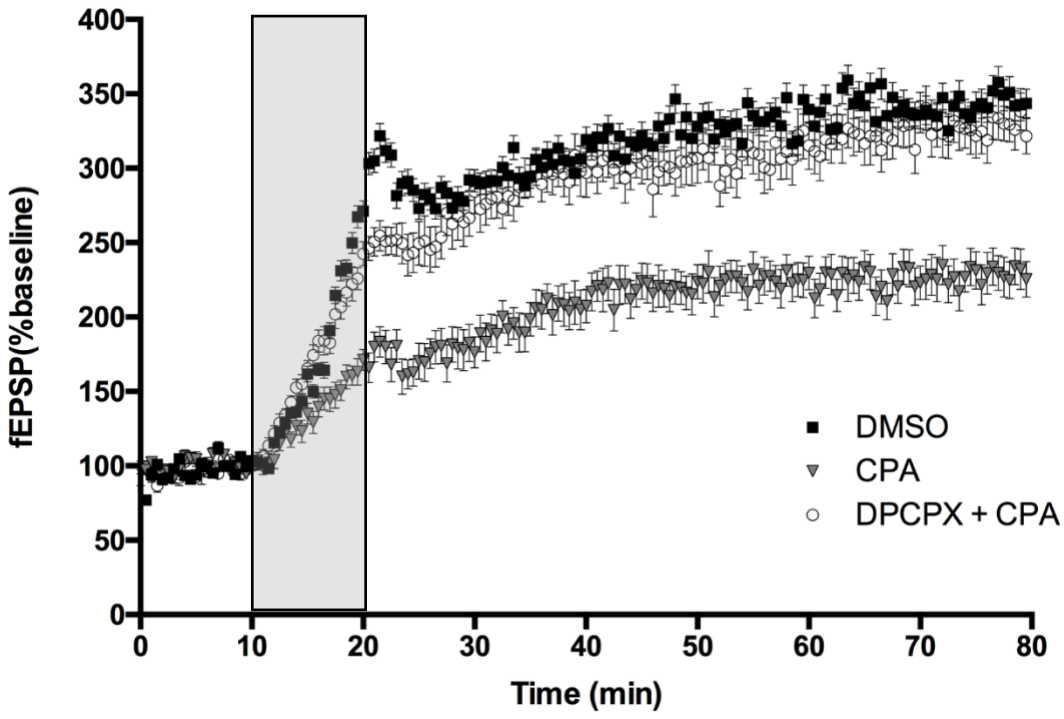
4.7 Long term potentiation (LTP) deficits after chronic A1R signalling

Previously in our lab, we have shown that prolonged stimulation of A1R leads to A1R-induced persistent synaptic depression (APSD). Also, activation of adenosine A1R leads to GluA2 and GluA1 AMPAR internalization (Chen et al. 2014). Studies in other labs have shown that inhibiting endocytosis of postsynaptic AMPARs prevents LTP decay, which could lead to converting of LTP decay into nondecaying LTP (Dong et al. 2015). Moreover, inhibition of AMPAR endocytosis in rat model of Alzheimer's disease improves memory and other cognitive functions (Dong et al. 2015). Thus, I decided to find out if the LTP deficits we observed involve adenosine A1R signalling through AMPARs. Rats were divided into three groups: Group A: Control group with DMSO intraperitoneal injection (i.p. injection) twice in 48 hours. Group B: CPA group with A1R agonist CPA (5 mg/kg) i.p. injection twice in 48 hours. Group C: DPCPX group with CPA (5 mg/kg) and A1R antagonist DPCPX (5 mg/kg) i.p. injection twice in 48 hours (20 min prior to CPA i.p. injections). Acute hippocampal slices were acquired from three different treatment groups on the following day after the two-day injection for electrophysiological analysis on their effect on LTP. Rats in CPA group showed significant LTP deficits when compared to the control group, whereas the rats in the DPCPX + CPA group had similar levels of LTP as the control group (Figure 4.7 A-C, summarized in D). These results suggested that chronic or longer-term adenosine A1R signalling is involved in inducing LTP deficits. In other words, persistent A1R activation could induce AMPAR internalization (Chen et al. 2014), which could underlie the observed impairment of LTP that can then be countered by co-administering the A1R antagonist DPCPX.

Figure 4.7.


A Control
B CPA-treated
C DPCPX + CPA
Baseline 1
Washout 2
Merge 1 + 2
Baseline 1
Washout 2
Merge 1 + 2
Baseline 1
Washout 2
Merge 1 + 2

D


fEPSP(%baseline)
Time (min)
DMSO
CPA
DPCPX + CPA
0
50
100
150
200
250
300
350
400
0
10
20
30
40
50
60
70
80

E

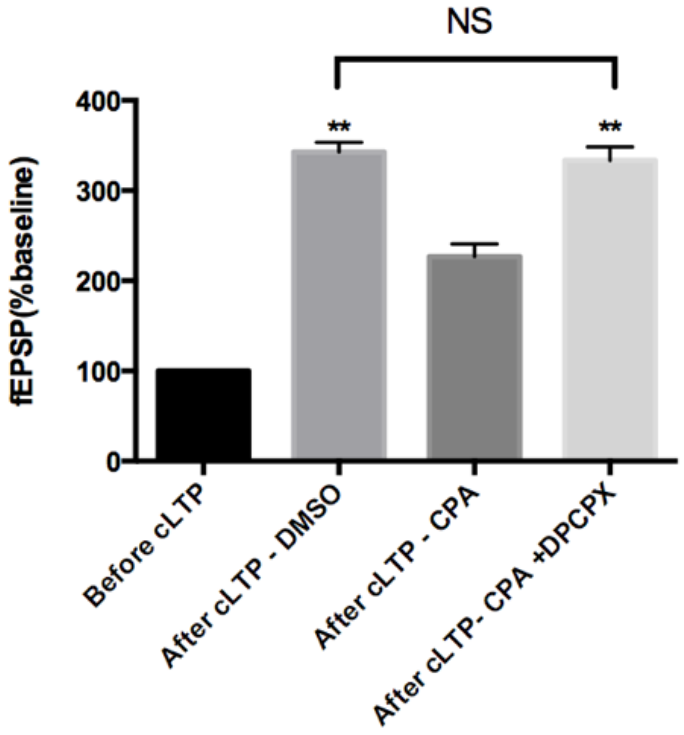
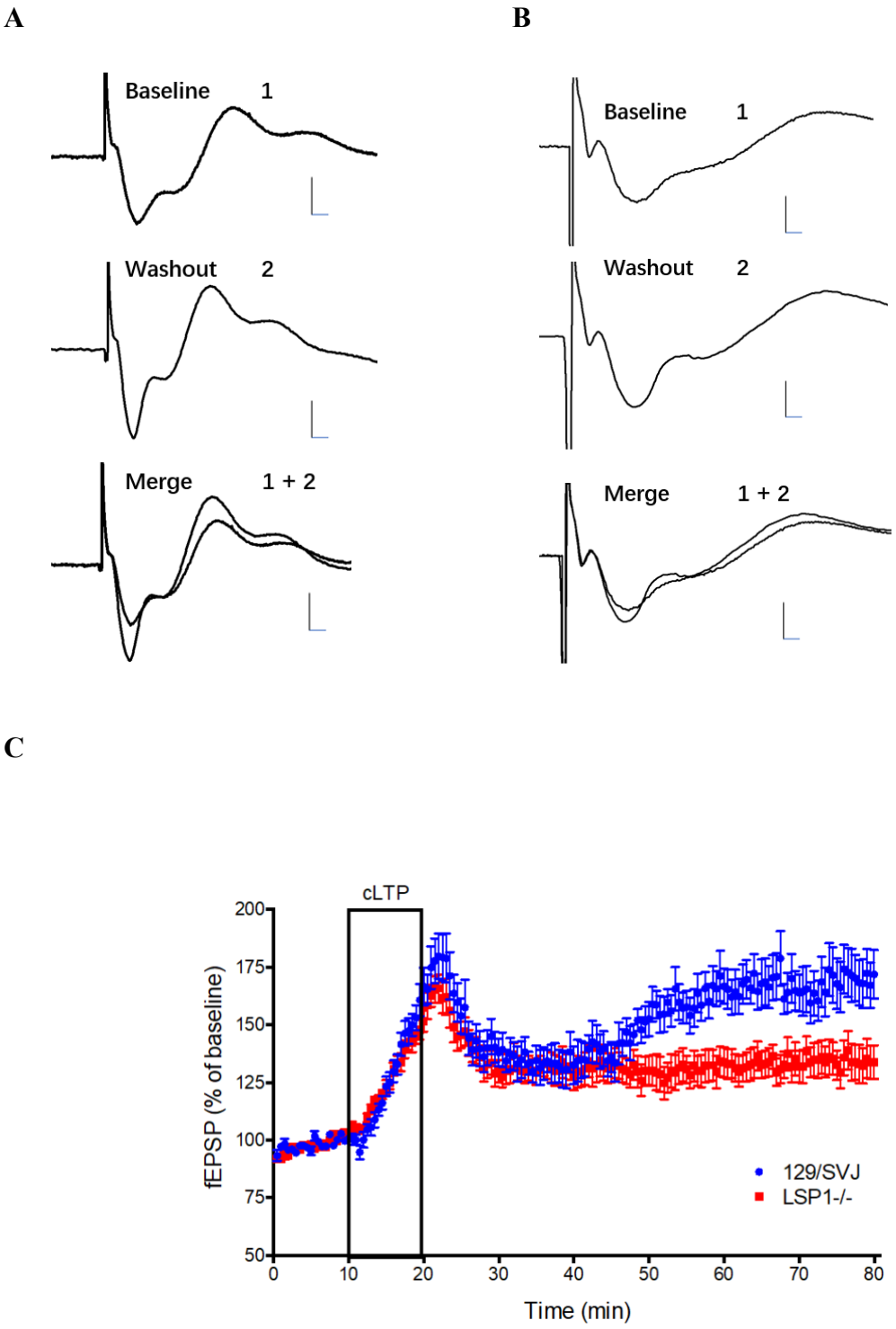

NS
**
**
400
300
200
100
0
fEPSP(%baseline)
Before cLTP
After cLTP - DMSO
After cLTP - CPA
After cLTP- CPA +DPCPX

Figure 4.7. LTP levels in rat hippocampus were decreased after CPA-induced A1R activation, which can be prevented by pretreatment with A1R antagonist DPCPX. A. Sample fEPSP traces for the DMSO group. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 60 minutes cLTP washout. (1 + 2) showing the overlay of the two traces. B. Sample fEPSP traces for the CPA group. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 60 minutes cLTP washout. (1 + 2) showing the overlay of the two traces. C. Sample fEPSP traces for the DPCPX + CPA group. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 60 minutes cLTP washout. (1 + 2) showing the overlay of the two traces. D. Summary time course plot showing mean fEPSP in three rat groups. Duration of chemical induction (with Rolipram and Forskolin) of LTP (cLTP) is denoted by the shaded region (total 10 min). E. Summary bar graph showing mean fEPSP values of three rat groups after washout of cLTP. Scale bars, 10 ms, 0.5 mV. Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: ** $p < 0.01$, NS $p > 0.05$. N = 12 recordings per group.

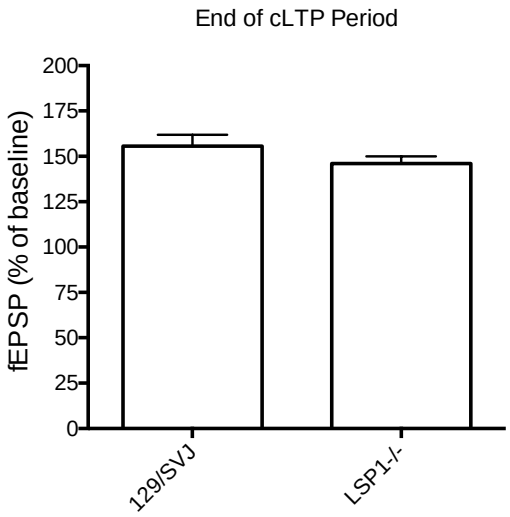
4.8 LSP-1 knockout mice showed LTP deficits when compared to wild type mice

Studies have indicated that adenosine is having pro-inflammatory effects by A1R signalling. Meanwhile, it also have anti-inflammatory effects through A2AR signalling (Nakav et al. 2008). Adenosine A1R is believed to regulate GluA2-containing AMPAR endocytosis through activation of p38 mitogen-activated protein kinase (p38 MAPK) (Chen et al. 2014). Interestingly, LSP1 is also known to serve as an important downstream substrate of p38 MAPK (Liu et al. 2005). Since A1R activation is involved in LTP impairment in rats, I aimed to provide a pilot study to establish a potential link between LSP1, adenosine A1R signalling, AMPARs and LTP induction and regulation. Our general hypothesis is that LSP1 may regulate endocytosis rates, and therefore, LSP1 knockout may increase the endocytosis of A1Rs and other receptors (Walther et al. 2006). My initial experiments aimed to investigate whether LSP1 knockout mice have LTP deficits, which is consistent with an enhanced endocytosis rate when LSP1 is absent (Walther et al. 2006). cLTP was induced in both LSP1 knockout mice and wild type mice. No significant difference was observed at the end of 10 min cLTP period, whereas I observed significant LTP deficit during the maintenance phase of LTP in LSP1 knockout mice (i.e., at the end of one-hour washout period) (Figure. 4.8 C). These results suggested that LSP1 is involved in the generation of LTP, however, further experiments still need to be conducted to find out whether LSP1 is normally involved in regulating A1R and AMPAR surface localization and synaptic plasticity in LSP1 knockout mice.

Figure 4.8.


A
Baseline 1
Washout 2
Merge 1 + 2
B
Baseline 1
Washout 2
Merge 1 + 2
C
cLTP
fEPSP (% of baseline)
200
175
150
125
100
75
50
0
10
20
30
40
50
60
70
80
Time (min)
129/SVJ
LSP1-/-

D


End of cLTP Period
fEPSP (% of baseline)
200
175
150
125
100
75
50
25
0
129/SVJ
LSP1-/-

E

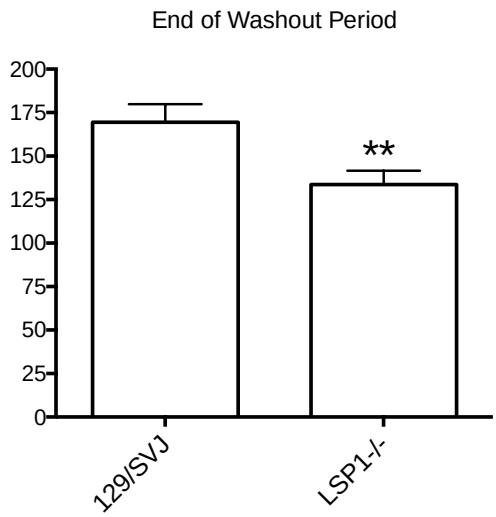

End of Washout Period
200
175
150
125
100
75
50
25
0
**
129/SVJ
LSP1-/-

Figure 4.8. LTP levels in mouse hippocampus were decreased after the one-hour LTP washout in LSP1 knockout mice compared to wild type mice, whereas no significant difference was observed at the end of cLTP perfusion period. A. Sample fEPSP traces for the wild type mice group. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 60 minutes cLTP washout. (1 + 2) showing the overlay of the two traces. B. Sample fEPSP traces for the LSP1 knockout mice group. (1) showing the average trace of the last 5 minutes of the 10 minutes baseline. (2) showing the average trace of the last 5 minutes of the 60 minutes cLTP washout. (1 + 2) showing the overlay of the two traces. C. Summary time course plot showing mean fEPSP in the two mouse groups. Note the biphasic response in the wild type mice, which is not observed in the LSP1 knockout mice. D. Summary bar graph showing mean fEPSP values of the two mouse groups at the end of cLTP perfusion period (10 min). E. Summary bar graph showing mean fEPSP values of the two mouse groups after the one-hour washout period after cLTP induction. Scale bars, 10 ms, 0.5 mV. Means ± SEM. Significance: ** $p < 0.01$. N = 12 recordings per group.

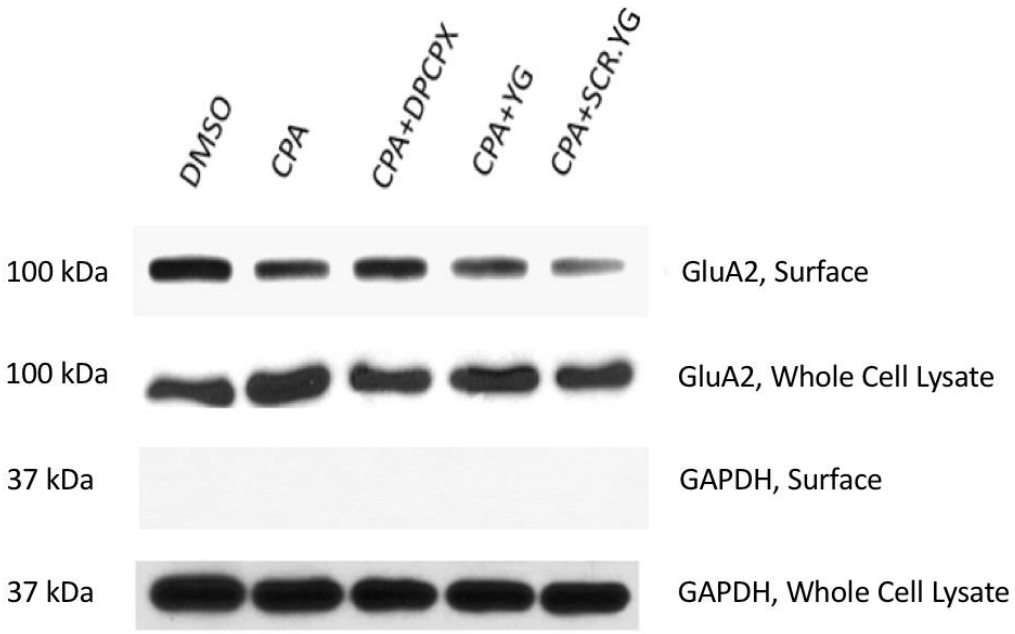
4.9 Short-term treatment of CPA decreased surface expression of GluA2, while prolonged treatment with CPA increased GluA2 surface expression

Since AMPAR trafficking is crucial in the process of neuron excitation, our lab has investigated whether A1Rs can functionally modify GluA2 and GluA1 AMPAR surface distribution *in vitro* and *in vivo*. Previously, we have shown that activation of A1Rs with CPA led to a significant reduction of GluA2 and GluA1 surface expression in hippocampal cultured neurons, which can be attenuated by DPCPX and "YG" peptide. That is, when this YG peptide is fused with Tat-domain peptide (which makes "YG" peptide become cell permeable), it allows selective inhibition of GluA2 clathrin-mediated endocytosis (Chen et al. 2014). In order to determine whether a similar inhibitory effect can be observed *in vivo*, I performed intraperitoneal (i.p.) injection of CPA with or without either DPCPX or YG peptide in adult rats. Following the 30-minute i.p. injections, rats were sacrificed, and hippocampal slices were subsequently immediately processed for biotinylation to detect the surface localization of GluA2 in these hippocampal slices. Results in Figure 4.9 A are consistent with our results in *in vitro* studies (Chen et al. 2014). To further determine the effect of A1R stimulation in chronic conditions, I performed another long-term experiment, in which I injected rats twice with the same treatments as I did in the 30 min groups in two consecutive days (48 hours treatment). Surprisingly, an increased GluA2 surface expression was observed, which highlights the difference between acute vs. chronic effects of A1R signalling in regulating synaptic plasticity-related molecular players. Future studies are needed to test whether the chronic A1R stimulation (as in the case of CPA i.p. injections for 2 consecutive days) results in an A1R-A2AR cross-talk that ultimately results in increased desensitization of A1R and a subsequent increased surface expression of A2ARs. Based on the preceding results presented so far, I predict that this chronic A1R stimulation will result in A2AR-mediated increase in GluA1/GluA2 surface insertion due to increased A2AR-mediated post-translational modification of either or both AMPAR subunits, most likely via increased phosphorylation at serine or threonine residues. Further studies are needed to test this hypothesis, and to further identify the downstream A1R signalling pathways that are important in contributing to these

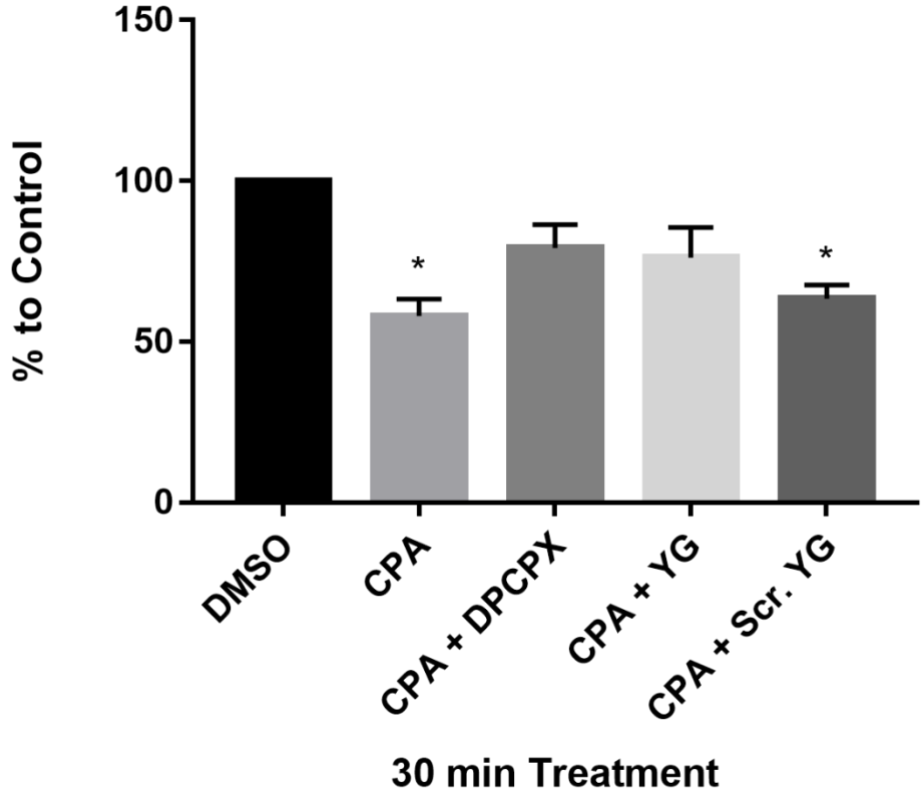
changes in GluA2/GluA1, A2AR and A1R in the early (acute) and late (chronic) phase of A1R stimulation that occurs in hypoxic/ischemic brain insults.

Figure 4.9.

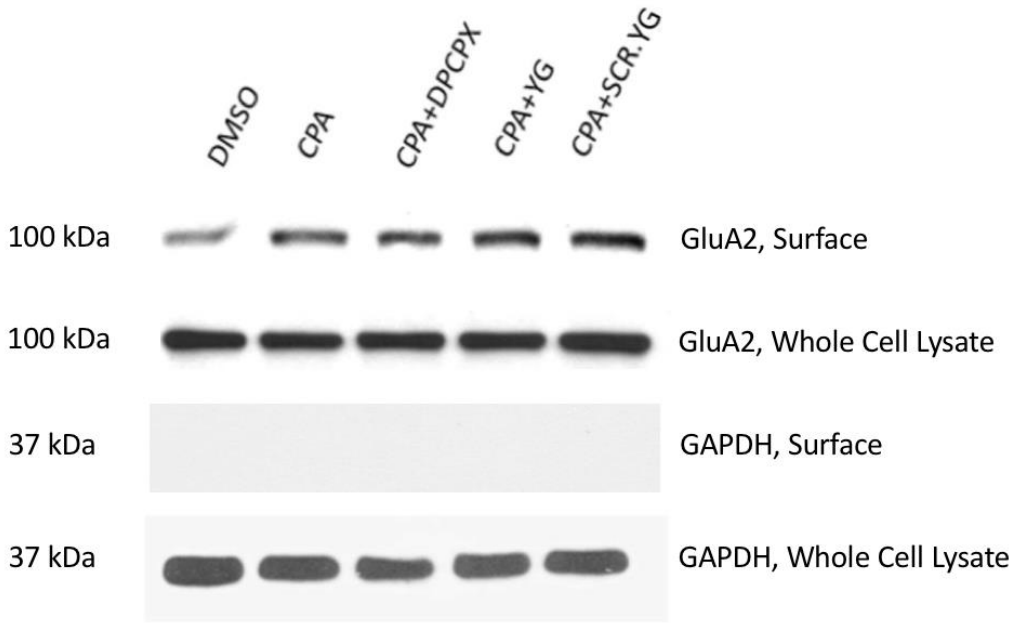
A


DMSO
CPA
CPA+DPCPX
CPA+YG
CPA+SCR.YG
100 kDa
GluA2, Surface
100 kDa
GluA2, Whole Cell Lysate
37 kDa
GAPDH, Surface
37 kDa
GAPDH, Whole Cell Lysate

B


150
100
50
0
% to Control
*
*
DMSO
CPA
CPA + DPCPX
CPA + YG
CPA + Scr. YG
30 min Treatment

C


DMSO
CPA
CPA+DPCPX
CPA+YG
CPA+SCR.YG
100 kDa
GluA2, Surface
100 kDa
GluA2, Whole Cell Lysate
37 kDa
GAPDH, Surface
37 kDa
GAPDH, Whole Cell Lysate

D

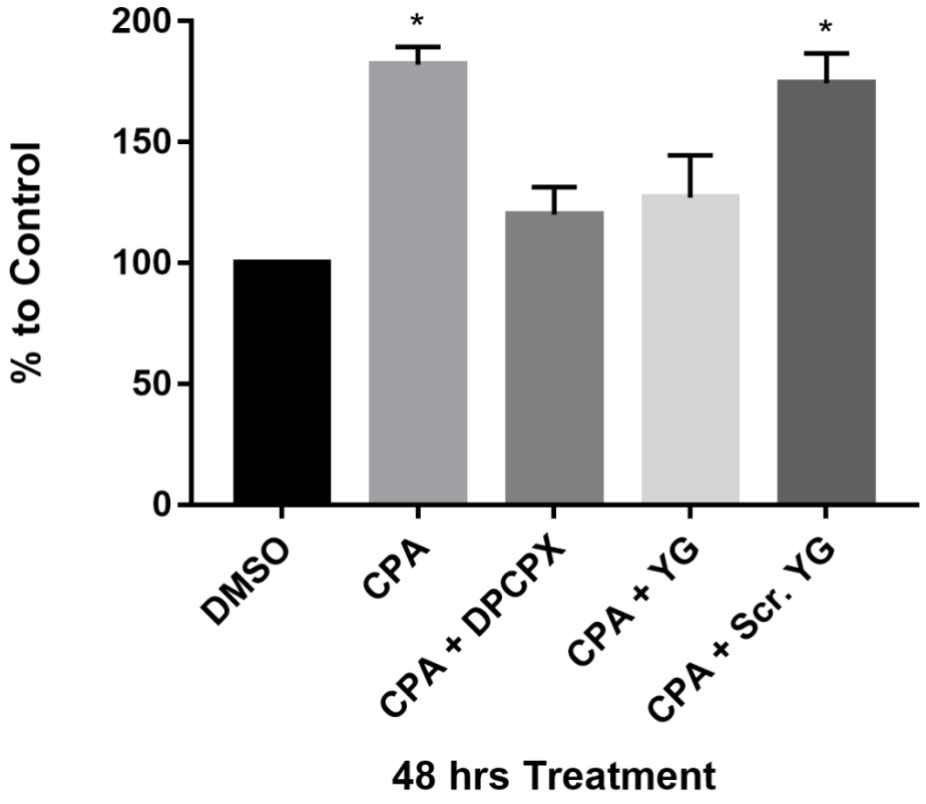

200
150
100
50
0
% to Control
*
*
DMSO
CPA
CPA + DPCPX
CPA + YG
CPA + Scr. YG
48 hrs Treatment

Figure 4.9. After injecting CPA (5 mg/kg) for 30 minutes, surface expression of GluA2 was downregulated when compared to the control group (i.e. injected with DMSO). This downregulation of GluA2 was attenuated by prior injection of DPCPX (5 mg/kg). Since the Tat-GluA2–3Y peptide particularly blocks clathrin-mediated GluA2 endocytosis, rats injected with CPA and YG peptide also showed similar results with the DPCPX group, whereas the scrambled YG peptide showed little effect on the reduction of GluA2 surface expression induced by CPA. However, in the 48-hour treatment group, CPA increased GluA2 surface expression. A. Western Blot data showing the different surface expression level of GluA2 after the acute treatment. A significant reduction of GluA2 surface expression was observed with CPA 30 minutes prior injection group when compared to control. Both injection of DPCPX and YG peptide attenuated the decrease of GluA2 surface expression, when no significant change was shown in the scrambled YG injected group when compared to the CPA injected group. B. Bar charts showing the level of biotinylated GluA2 in different treatment group after normalizing on the respective whole hippocampal slice cell lysates. C. Western Blot data showing the different surface expression level of GluA2 after the chronic treatment. A significant increase of GluA2 surface expression was observed with CPA 48 hours injection group when compared to control. Both injection of DPCPX and YG peptide attenuated the increase of GluA2 surface expression, when no significant reduction was shown in the scrambled YG injected group. Means ± SEM. Significance: * $p < 0.05$. N = 4 from four independent experiments

5. Discussion

5.1 Adenosine A1 and A2A receptor cross-talk

Previous studies in our lab (Stockwell and Cayabyab, unpublished) have demonstrated that blocking A1R or A2AR alone can reduce the adenosine-induced post-hypoxia synaptic potentiation (APSP), which strongly suggested a potential cross-talk between A1R and A2AR that could contribute to enhanced APSP levels and the associated neuronal damage after hypoxia treatments in the rat hippocampal brain slices. However, the mechanism underlying the interaction of A1 and A2A receptors remains to be further explored. Studies have shown that selective activation of A2ARs with CGS 21680 generated an attenuation of the A1R binding in rat hippocampus (O'Kane and Stone 1998). Since the A2AR agonist CGS 21680 was shown to decrease the ability of the A1R agonist CPA to inhibit neuronal excitability, Cunha's lab previously proposed that a functional interaction between A1R and A2AR exists (Lopes et al. 1999). Whether the A2AR attenuation of A1R binding of CPA or adenosine is a result of A1R and A2AR cross talk is still unknown. Preliminary investigations in our lab showed that Casein Kinase 2 (CK2), a serine/threonine protein kinase, is downregulated in our pial vessel disruption (PVD) stroke model (Chen and Cayabyab, unpublished). Moreover, our lab reported that in the PVD stroke model, A1Rs are downregulated after the PVD ischemic insult, whereas A2ARs are upregulated (Chen et al. 2014). Considering the fact that CK2 negatively regulates A2AR desensitization (Rebholz et al. 2009), we hypothesize that CK2 is intimately linked to enhancing A2AR surface expression. Future studies are needed to further test whether chronic A1R stimulation with CPA mimics the effects of PVD, by causing a decreased CK2 expression and, hence, an increased A2AR surface expression.

5.2 Adenosine, adenosine receptors and neuroprotection

As a neuromodulator, adenosine not only contributes to neuroprotection by inhibiting excitatory neurotransmission but it could also mediate neuroprotective intermediary metabolism and signalling, which is believed to be the primary mechanism underlying its protective effect in non-brain tissue (Cunha 2001). Although A1Rs were commonly recognized

as a neuroprotective modulator in the brain, due to the fact that they reduce glutamate release and cause neuron hyperpolarization, A1Rs are found to be downregulated in chronic noxious conditions. During hypoxia, prolonged stimulation of A1Rs leads to desensitization of A1Rs which could underlie the inhibition of synaptic transmission regulated by the internalization of A1R. Carvalho's lab has observed that blockade of A1Rs attenuated the hypoxia-induced decrease of energy charge and prevents the recovery of metabolic alterations during the normoxic period (Duarte et al. 2016), which is different with the neuroprotective effect I observed in the present study. However, the duration of hypoxia is different in their study, in which they used a 90-min hypoxia treatment to mimic stroke conditions. Further *in vivo* studies on animal stroke models are needed to determine which kind of hypoxia treatment could more accurately represent the human stroke conditions. Currently research in our lab has shown some promising results with A1R antagonist's neuroprotective effect in the PVD stroke model which is in line with the observations in the present study. A2AR antagonists have also shown their neuroprotective effects in ischemic brain damage and Parkinson's disease, which has not been thoroughly studied in my project. Molecular studies have provided some evidence on the involvement of adenosine receptors and metabotropic glutamate type 1 and type 5 receptors in neurodegenerative diseases (Canals et al. 2003; Ferre et al. 2002; Nishi et al. 2003), which encourages us to further explore the interactions between A1R and A2AR. The mechanisms behind how A1R and A2AR control neuroprotection remains to be established in further investigations. This project has provided some evidence that the blockade of A1Rs could contribute to neuroprotection by regulating synaptic transmission through glutamate receptors, i.e., by reducing the expression and function of CP-AMPARs that have been shown in my studies to promote neuronal damage. Further studies need to be conducted in adenosine receptors' effects on metabolism and neurogenesis. The potential mechanism between LSP1, p38 MAPK and A1R implicated the further crucial role of adenosine receptors in mediating neuroinflammation. Even though the complete pathway of adenosine signalling still remains unclear, it is evident to suggest that targeting adenosine receptors will provide a novel strategy for the treatment of multiple neurological disorders.

5.3. Potential therapeutic target for stroke

Clinical trials for direct antagonism of the NMDAR as a stroke therapeutic target have been disappointing. Two main reasons have been pointed out in recent studies. One is that some NMDAR antagonists interfere with some important physiological NMDAR functions (Roesler et al. 2003), while other major reason is that most of the agents have a narrow therapeutic window (Ikonomidou and Turski 2002). The unsuccessful translation of neuroprotective drugs from bench to bed side for patients suffering from ischemic stroke over the years has strongly suggested that new strategies are eagerly required for both animal stroke models and in the clinical realm. Researches have suggested that AMPA receptors have a more significant effect in neurodegeneration process of hippocampal CA1 neurons than NMDARs (Buchan et al. 1993; Chang et al. 2012; Noh et al. 2005; Schielke et al. 1999). According to electrophysiological studies (Buldakova et al. 2007), Ca^{2+}-permeable AMPAR antagonists increased synaptic transmission at Schaffer collateral to CA1 region during hypoxic conditions. Moreover, some blockers selective for Ca^{2+}-permeable AMPARs, such as IEM 1460, have been shown to substantially reduce post ischemic neurodegeneration in the CA1 region, which strongly indicates a crucial role for Ca^{2+}-permeable receptors as a potential therapeutic target for treating delayed neuronal death after hypoxic-ischemia insult (Schlesinger et al. 2005; Xiong et al. 2009). In my current research project, some Ca^{2+}-permeable AMPAR antagonists have not shown significant neuroprotective effects when administered after the hypoxic insult, which is consistent with the suggestion that drugs based on this mechanism failed in clinical trials. However, Perampanel, the clinically approved drug used for treating partial seizures and generalized seizures for people older than 12 years old is showing beneficial effects when applied both during hypoxia and post-hypoxic conditions. It is intriguing to find out why Perampanel is having a different effect on post-hypoxia potentiation when compared to other AMPAR antagonists. One possible reason is that Perampanel increases $GABA_AR$-mediated inhibitory neurotransmission in the rat hippocampus by a mechanism which is not mediated by AMPARs and kainate receptors. Studies on GABA receptor regulated synaptic transmission in the cerebellum observed that CNQX, a commonly used non-NMDA receptor antagonist, can lead to the up-regulation of inhibitory post synaptic currents (IPSCs) (Brickley et al. 2001).

Interestingly, GYKI 52466, an relatively more selective AMPA receptor antagonist produced no increase in IPSC frequency (Wilding and Huettner 1995). Since higher dosage (3 μM) of Perampanel could also completely block synaptic transmission in the stratum radiatum of the CA1 region (Rogawski and Hanada, 2013; also confirmed in our lab, data not shown), which is similar to my results with CNQX which completely prevented the baseline fEPSPs and APSPs, it is plausible to suggest that Perampanel could also be mediating an allosteric modulation of $GABA_AR$-mediated synaptic transmission. Ongoing investigations in our lab have already started to test whether Perampanel's neuroprotective effects on the post-hypoxia neuronal damage can be prevented when $GABA_AR$-mediated inhibitory postsynaptic transmission is blocked by Bicuculline. The core structure of Perampanel has a 2,3'-bipyridin-6'-one, which could be a distinguishing feature of Perampanel from other AMPA receptor antagonist classes (Rogawski and Hanada 2013); this core structure could account for its stronger neuroprotective effects over other AMPAR inhibitors during hypoxic/ischemic brain damage.

5.4 Distinguish among different AMPAR subtypes.

Diversity in the functional AMPAR subtypes have shown their importance in regulating AMPAR signalling in the CNS. In my current study, I applied three different kinds of Ca^{2+} permeable AMPAR antagonists. However, in order to elucidate whether there is a GluA1-containing AMPAR re-insertion during the reperfusion period which leads to different neuroprotective effects, better research tools are needed to distinguish GluA1-containing AMPARs and GluA3-containing AMPARs. In early observations, researchers reported that Philanthotoxin 74, when tested at concentrations of 100 and 500 μM, blocked 80% of GluA1/GluA2 AMPARs but only blocked 10% of GluA2/GluA3 AMPARs (Nilsen and England 2007). This study suggested that Philanthotoxin-74's potential role in distinguishing different combination of subtypes among AMPAR populations. However, conflicting results have been reported in Poulsen's lab, showing that the marginal influence of the AMPAR subunits affects the affinity of Philanthotoxin-74, which might lead to difficulties in distinguishing their functional differences. (Poulsen et al. 2013). At present, the best way to

investigate changes in GluA1-containing AMPARs is through biochemical methods. Although I provided some preliminary biochemical evidence that GluA2 surface expression could be altered in different ways depending on the time of exposure to A1R agonists *in vivo*, clearly more studies and other biochemical/molecular tools will be needed to address whether different classes of AMPAR antagonists can differentially affect the subunit compositions of functional AMPARs in normal and hypoxic/ischemic conditions.

5.5 Kainate receptor in synaptic transmission

Another important glutamate receptor that has not been researched in this study is kainate receptor. Since numerous AMPAR agonists and antagonists can also interact with the kainate receptors, AMPARs and kainate receptors are commonly classified by scientists as non-NMDA receptors. According to the current knowledge of kainate receptors in the neuroscience field. The roles for kainate receptors in physiological process for neurotransmission have been poorly studied, due to the deficiency of selective antagonist and agonist of kainate receptors. Studies showed that GYKI 53655 can antagonize AMPARs, while it had no significant effect on kainate receptors (Wilding and Huettner 1995). With the application of GYKI 53655, scientists have revealed that certain synaptic responses during the interaction between hippocampal CA1 region and Schaffer collaterals are mediated by kainate receptors (Frerking et al. 1998). Application of kainate has been shown to reverse the suppression of EPSPs and EPSCs in CA1 (Kamiya and Ozawa 1998). These results have suggested that kainate receptors have a widely expression in the CNS and may have significant functions in mediating synaptic functions. Whether these kainate receptors are similarly regulated by A1R and A2AR signalling remains to be established.

5.6 Ca^{2+}-permeable AMPAR trafficking.

Ca^{2+}-permeable AMPARs have now shown their importance in synaptic transmission and synaptic plasticity, due to the observation of the rapid subunit-specific trafficking of synaptic AMPAR. The trafficking of AMPARs have been well studied, however, little research has been

conducted on CP-AMPARs. Researches have already shown the importance of GluA1-dependent mechanisms on the LTP expression. In this project, I have indicated the relationship between AMPARs and the level of LTP. Meanwhile, in the electrophysiology studies, I hypothesis the insertion of CP-AMPARs to the cell surface during the washout period. Although LTP were commonly considered as GluA1-dependent, there is still a possibility that insertion of CP-AMPARs contributes to other forms of synaptic plasticity. Moreover, it is essential to investigate the upstream activities of different AMPAR subunits, and the interactions between AMPAR accessory proteins with CP-AMPAR expression. The importance of CP-AMPAR is not only evident in the hippocampal neurons, but also in the lateral amygdala, due to their similarities in CP-AMPAR trafficking. The present findings of CP-AMPAR's crucial role in the hippocampus may further implicate the roles of adenosine-mediated CP-AMPAR induction and abnormal AMPAR functions in drug addiction, depression, short term memory loss and their potential therapeutic role in brain ischemia. Further investigation on the mechanisms of the observation at synapses might uncover potential therapeutic targets. The most valuable findings in this study are that late Perampanel administration significantly prevents the APSP and other CP-AMPAR blockers like NAPSM and IEM 1460 will also provide neuroprotection when applied at the time of the hypoxic insult. These findings suggest the GluA2-lacking AMPARs that are expressed at CA1 synapses during chronic phases of ischemia, do having a significant effect in neurodegeneration in the rat hippocampal CA1. Additionally, my results also suggest that to increase neuroprotection, an early intervention with Perampanel can block the majority of GluA2-lacking AMPARs. GluA2-lacking AMPARs are normally having a relatively low expression in the rat hippocampal slices in most conditions, but their surface expression can be dramatically enhanced during reperfusion. CP-AMPARs are also suggested to be associated with other neurological disorders and brain insults. In the past decade, researchers have implicated CP-AMPARs as having important roles in seizures (Rogawski and Hanada 2013), Alzheimer's and Parkinson's disease (Cunha 2005; Whitehead et al. 2017). My study in this area has provided further evidence for CP-AMPAR's involvement in hypoxia-induced delayed neuronal death in the hippocampal brain region and suggests that the application of CP-AMPAR antagonists need to be revisited as some agents, like Perampanel,

could eventually lead to an effective treatment in post-stoke neuronal injury and other neurological disorders.

5.7 LSP1, p38 MAPK and synaptic plasticity

In early studies, scientist have shown that p38 MAPK signalling is important for the induction of synaptic plasticity through the induction of NMDAR-induced long term depression (LTD) and mGluR-induced LTD. (Thomas et al. 2004; Collingridge et al. 2010). mGluR-induced LTD is depending on the activation of p38 MAPK. In previous studies (Chen et al. 2014; Stockwell et al. 2016), our lab has shown that stimulation of A1R can lead to the activation of p38 MAPK, which then leads to the internalization of GluA2 and GluA1. This could suggest a potential mechanism for p38 MAPK-dependent mGluR-induced LTD. However, the direct downstream target of p38 MAPK underlying LTD still needs to be investigated. Base on the fact that LSP1 serves as a important downstream substrate of p38 MAPK and the observed deficiency of LSP1 in LSP1 knockout mice causing LTP deficits in the present study, it is reasonable to suggest that LSP1 is also affecting in this mGluR-induced LTD. When comparing the results from mouse and rat cLTP studies in this project, I observed a decreased level of LTP in mouse hippocampal slices after the induction of LTP. Some labs have suggested an age-related developmental loss of LTP in mouse visual cortex, (Kirkwood et al. 1997; Yoshimura et al 2003), which may underlie this deficiency of LTP in 12-15 week old mice. Since mouse hippocampal slices are much smaller than those of rats, and I used the same bi-polar stimulation electrode for rat and mouse studies, the difference in the results I observed in mouse and rats (and those of others) might due to inaccurately stimulating the mouse CA1 region. Further studies are needed to explain this decrease of synaptic transmission after the induction of LTP.

6. Conclusion

Both adenosine A1Rs and A2ARs are involved in the post-hypoxia potentiation, which indicates delayed neuronal death in the hippocampus induced by hypoxic insult. Post-hypoxia neurotransmission in the rat CA1 hippocampus is mostly regulated by AMPA-ergic receptors, instead of NMDA receptors.

A priority of this investigation was to identify the effects of glutamate receptors in order to find out potential therapeutic targets for ischemic stroke. Similar to NMDAR antagonists, Ca^{2+}-permeable AMPAR blockers in this study are only effective when applied at the early stage of insult. Perampanel, on the other hand, could potentially provide neuroprotection in hypoxic conditions, and could potentially be effective even during ischemia/reperfusion periods. Further studies aimed at determining how Perampanel mediates this increased neuroprotection are warranted, including studies of levels of neuroinflammation, levels of reactive oxygen production, and levels of Ca^{2+}-induced excitotoxicity.

LTP impairment occurs when global activation of A1R is induced, which can be countered by A1R antagonists. The mechanisms underlying A1R-mediated AMPAR endocytosis and synaptic plasticity changes still need further investigation. Adenosine signalling, and the different glutamate receptor functions studied in this project, may ultimately provide a more comprehensive understanding as a basis for an effective rationale to investigate and identify future therapeutic strategies for ischemic stroke.

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Anon. n.d. "HS_StrokeReport2017_EN."

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