Effect of human equilibrative nucleoside transporter 1 (hENT1) and ecto-5'nucleotidase (eN) in adenosine formation by neurons and astrocytes under ischemic conditions.

By

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Abstract

Adenosine (ADO) is an endogenous neuroprotectant. Under ischemic conditions ADO levels rise in the brain up to 100-fold. ADO in the brain is dependent on the movement across cell membranes by equilibrative nucleoside transporters (ENT) or produced from membrane bound ecto-5' nucleotidase (eN). We used transgenic neurons with neuronal specific expression of human ENT1 (hENT1) and eN knockout (CD73 KO) astrocytes. The aim of this research was to determine the role of ENT1 and eN in ADO release from ischemic-like conditions in primary cultured neurons, astrocytes or co-cultures.

Neurons primarily release intracellular ADO via ENTs; this effect was blocked by transporter inhibitor, dipyridamole (DPR). Astrocytes primarily convert ADO extracellularly from eN; this effect was with eN inhibitor α, β-methylene ADP (AOPCP). Combined neuron and KO astrocytes produced less ADO, extracellular ADO was inhibited by DPR but not AOPCP. Overall these results suggest that eN is prominent in the formation of ADO but other enzymes or pathways contribute to rising ADO levels in ischemic conditions.

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Abbreviations

[^{3}H]	tritium
µg	microgram
µL	microliter
µM	micromolar
2DG	2-deoxy glucose
ACh	acetylcholine
ADA	adenosine deaminase
ADE	adenine
ADO	adenosine
ADP	adenosine diphosphate
AK	adenosine kinase
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-propionate
ATP	adenosine triphosphate
AN	adenine nucleotides (ie. ATP, ADP, AMP)
AOPCP	α, β-methylene ADP
cAMP	cyclic AMP
CB_1	cannabinoid receptor type 1
CD73	ecto 5' nucleotidase
cGMP	cyclic GMP
CNS	central nervous system
CNT1	concentrative nucleoside transporter 1
CNT2	concentrative nucleoside transporter 2

CNT3	concentrative nucleoside transporter 3
CPA	N6-cyclopentyladenosine
CPT	8-cyclopentyltheophylline
D_2	dopamine receptor subtype 2
DPCPX	8-cyclopentyl-1, 3-dipropylxanthine
DPM/min	disintegrations per minute
DPR	dipyridamole
DIV	days *in vitro*
DMEM-F12	Dulbecco's modified Eagle medium F12
ecto-ADA	adenosine ecto-deaminase
eN	ecto 5'-nucleotidase
E-NTPDase	ectonucleoside triphosphate diphosphohydrolases
e-NPP	ectonucleotide pyrophosphatase/phosphodiesterases
ENT1	equilibrative nucleoside transporter 1
ENT2	equilibrative nucleoside transporter 2
ENT3	equilibrative nucleoside transporter 3
ENT4	equilibrative nucleoside transporter 4
ei	equilibrative insensitive
es	equilibrative-sensitive
ENT	equilibrative nucleoside transporter
FBS	fetal bovine serum
fEPSP	field excitatory post synaptic potential
GABA	gamma-aminobutyric acid
GD	glucose deprivation

G_i / G_o	inhibitory G protein
G_{olf}	olfactory G protein
G_s	stimulatory G protein
GPI	glycosyl-phosphatidyl-inositol
hENT1	human equilibrative nucleoside transporter 1
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HX	hypoxanthine
IB–MECA	N6-(3-iodobenzyl) adenosine-5'-N-methyluronamide
IMP	inosine monophosphate
INO	inosine
IP_3	inositol 1, 4, 5-triphosphate
kBq	kilobequerel
K_d	dissociation constant
kg	kilogram
K_i	inhibitory constant
K_m	Michaelis-Menton constant
KO	knock-out
LTD	long-term depression
LTP	long-term potentiation
mGlu5	metabotropic glutamate receptor subtype 5
min	minutes
mL	milliliters
mmol	milimolar
mOsm	miliosmole

MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NAD^+	nicotinamide adenine dinucleotide
NBMPR	S-(4-nitrobenzyl)-6-thioinosine
NBTI	nitrobenzylthioinosine
nM	nanomolar
NMDA	N-methyl D-aspartate
NO	nitric oxide
NR1	NMDA receptor subunit 1
NR2B	NMDA receptor subunit 2B
nonREM	non rapid eye movement
NTPase	nucleoside triphosphate diphosphorylase
OGD	oxygen glucose deprivation
PAP	prostatic acid phosphatase
PCP	phencyclidine
PNP	purine nucleoside phosphorylase
PD	Parkinson's disease
PLA_2	phopholipase A2
PLC	phospholipase-C
pmol	picomoles
pmol/mg	picomole per milligram
PNP	purine nucleoside phosphorylase
REM	rapid eye movement
rpm	revolutions per minute
RT-PCR	real-time polymerase chain reaction

SAH	S-adenosylhomocysteine
SEM	standard error of the mean
SD	standard deviation
SWS	slow wave sleep
Tg	transgenic
TLC	thin layer chromatography
TM	trans-membrane
TNAP	tissue-nonspecific alkaline phosphatase
tPA	tissue-type plasminogen activator
UV	ultraviolet
VNUT	vesicular nucleotide transporter
Wt	wild type

Chapter 1: Introduction

1. 0. Adenosine

In all living cells purines are fundamental components for cellular activity. Adenosine triphosphate (ATP) is the unit of energy used by cells to do work and adenine (ADE) is a nucleobase constituent of nucleic acids. Purines have been identified for intracellular and extracellular signaling. Extracellular signaling by ATP has been identified and is mediated by purinergic receptors, whereas intracellular signaling is mediated by cyclic adenosine monophosphate (cAMP). Adenosine (ADO) is another signaling molecule with diverse physiological effects. The actions of ADO are mainly modulating excitability in the heart and brain. ADO signaling is also involved in vasodilation, angiogenesis, insulin secretion, lipolysis, allergy and inflammation (Relavic and Burnstock 1998; Dunwiddie and Masino 2001).

ADO also behaves as a neuromodulator within the central nervous system (CNS). ADO modulates many brain functions including arousal and sleep, locomotion, anxiety, cognition and memory (Ribeiro et al., 2003). At rest ADO can be found in the extracellular environment in low nanomolar (30-300nM) concentrations (Rudolphi and Schubert 1997). Severe metabolic stress associated with hypoxia, ischemia, brain trauma and inflammation lead to sudden increases in extracellular ADO levels up to 10-50 µM (Hagberg et al., 1987).

ADO cannot be described as a neurotransmitter; ADO is not stored or released through synaptic vesicles (Phillis et al., 2001). ADO exerts its effects extracellularly by modulating synaptic transmission by inhibiting or facilitating transmitter release and by

hyper- or depolarizing neurons (Cunha 2001a). The dysfunction of ADO in the CNS has been implicated in variety of neuropathies including stroke, epilepsy, Parkinson's disease, neuropathic pain, depression, anxiety, and schizophrenia (Sebastião et al, 2009).

1.1. Adenosine Receptors

The actions of adenosine are mediated through cell surface receptors from the purinergic receptor (P1) family. There are four distinct types of G-protein coupled receptors, A_1, A_{2A}, A_{2B} and A_3. The A_2 receptors are further divided, by their affinity for ADO. The four ADO receptors can increase or decrease adenylyl cyclase activity. The inhibitory receptors, A_1 and A_3, decrease adenylyl cyclase which decreases cAMP. However A_{2A} and A_{2B} are excitatory and increase adenylyl cylcase and thus increase cAMP activity (Burnstock 2007a). The cloning of ADO receptors in several mammalian species has allowed characterization of the distribution of ADO receptors in various brain regions. In the CNS, the neuromodulatory effects of ADO are mainly exerted by A_1 and A_{2A} high affinity receptors (Cunha 2001; Gomes 2001; Ribero et al, 2003).

1.1.1. A_1 Receptors

The receptor A_1 has high affinity for ADO compared to the other subtypes and is highly expressed in neurons of the regions of the cortex, hippocampus, cerebellum and dorsal horn of the spinal cord (Ribeiro et al., 2003). Cell type expression of A_1 has been found in neurons (Londos et al., 1980; van Calker et al., 1979), astrocytes (Biber et al,

1997), microglia (Gebicke-Haerter et al, 1996), and oligodendrocytes (Othman et al., 2003).

In neuronal circuits, A_1 receptors are inhibitory at excitatory synapses at pre and post synaptic terminals (Cunha 2001). Activation of the A_1 receptor recruits the inhibitory G proteins (G_i and G_o) and negatively impacts on adenylyl cyclase function (Linden 2001). A_1 activation causes hyperpolarization of neurons by activating K^+ channels and inactivates several types of presynaptic Ca^{2+} channels. This results in decreased release of neurotransmitters, including glutamate, acetylcholine (ACh), dopamine, noradrenaline and serotonin. During ischemic or hypoxic insult, activation of the A_1 receptors inhibits glutamate release presynaptically and inhibits NMDA receptors post synaptically (de Mendonça et al., 1995). All of these processes, through the activation of A_1 receptors depress neuronal excitability and metabolism (Ruldophi et al., 1992; Bischofberger et al., 1997; Fredholm et al, 2005). Enhancing A_1 receptor activation with elevated extracellular ADO levels could potentiate neuroprotection in the brain during hypoxic or ischemic insult.

1.1.2. A_{2A} Receptors

The high affinity A_{2A} receptor is expressed in restricted regions, including the immune tissues, platelets, vascular smooth muscle and endothelium. High abundance of A_{2A} receptor mRNA has been found in spleen, immune tissues, eye, skeletal muscle, heart, lung, bladder and uterus (Dixon et al., 1996; Peterfreund et al., 1996). In the CNS, A_{2A} is highly expressed in dopamine rich areas such as striatum, globus pallidus, nucleus accumbens, and tuberculum olfactorium (Jarvis et al., 1989). High levels of A_{2A} receptor

expression are found in the striatum and are co-localized with dopamine (D_2) receptors in GABAergic neurons containing enkephalin (Fink et al., 1992).

A_{2A} receptor activation is excitatory; activation of stimulatory G (G_s) protein leads to stimulation of adenylyl cyclase in peripheral tissues. However, in the striatum, G_{olf} is used to stimulate adenylyl cyclase (Kull et al., 2000). Stimulation of the A_{2A} receptors activates Ca^{2+} ion channels, Na^+ channel and NMDA receptor function in the striatum (Stella et al., 2002; Nash and Brotchie, 2000; Norenberg et al., 1998). A_{2A} receptors modulate GABA neurotransmitter release in conjunction with A_1 receptors (Lopes et al., 2002) and have also been implicated in glutamate, ACh and serotonin neurotransmitter release (Cunha, 2001).

In contrast to A_1 receptor-mediated neuroprotection, A_{2A} receptor agonists contribute to neuronal injury in ischemic and excitotoxicity models (Gao and Phillis 1994; Rudolphi et al., 1992b). Neuronal death from excessive neurotransmitter release of excitatory amino acids is thought to be facilitated by A_{2A} receptor activity. The facilitating effect of A_{2A} agonists has been shown to enhance excitatory effects of glutamate under ischemic conditions. Thus, neuroprotection in various animal models of cerebral ischemia can be shown using A_{2A} antagonists such as SCH 58261. In disease models, A_{2A} receptor is implicated in the pathology in certain neurological disorders such as Parkinson's, Huntington's and Alzheimer's diseases, schizophrenia, phobias, depression, and bipolar disorders.

1.1.3. A_{2B} Receptors

The A_{2B} receptors are found on all cells and have low affinity for ADO. The A_{2B} receptor is widely distributed in the brain albeit at low expression levels (Daly et al,. 1983; Dixon et al., 1996). The A_{2B} receptor is excitatory, coupling positively to adenylyl cyclase through G_q and G_s proteins (Feoktistov et al., 1994; Linden 1999). In the CNS, A_{2B} receptor may be involved with neurodevelopment of dorsal spinal cord axons and may interact with inflammatory cytokines associated with traumatic brain injury and ischemia (Corset et al., 2000). Also the A_{2B} receptor may inhibit taurine release from glial cells of the neurohypophyses. Taurine may play a role in cell osmoregulation and could potentially be implicated in the pathophysiology of olfactory, auditory, visual systems development and long term potentiation (LTP) in the striatum (Pierson et al., 2007; Warskulat et al., 2007).

In the periphery, high expression levels can be found in the caecum, colon and bladder (Stehle et al., 1992). Moderate expression of A_{2B} receptors is seen in lung, blood vessels and eye; low levels of A_{2B} receptor expression include adipose tissue, adrenal gland, kidney, liver, ovary and pituitary gland (Fredholm, 2000). The A_{2B} receptor positively impacts on levels of adenylyl cyclase and increases intracellular calcium signaling (Feoktistov et al., 1994). Physiological roles of A_{2B} receptors have been identified in airway smooth muscle, fibroblasts, astrocytes, gastrointestinal tract, and blood vessels. A_{2B} receptors may be implicated in degranulation of immune cells and may play a role in allergy and inflammatory disorders.

1.1.4. A_3 Receptors

The A_3 receptor is known to show a species-specific tissue distribution and expression is higher in sheep >humans > rodents. Pharmacological profile of the A_3 receptor in humans is similar to sheep than rat homolog (Linden et al., 1993; Salvatore et al., 1993). The peripheral distribution of A_3 receptor is extensive and can be found in testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon and eye (Linden et al., 1993; Salvatore et al., 1993; Dixon et al., 1996). In rat, expression levels of A_3 receptors are highest in testis and mast cells. However in sheep, highest expression was found in the lung, spleen, pars tuberalis and pineal gland (Linden et al., 1993; Salvatore et al., 1993). In humans, A_3 receptor distribution is widespread, with highest expression in the lung and liver; in contrast the lowest expression is in the aorta and brain (Salvatore et al., 1993). In the CNS, the A_3 receptor is expressed on neuronal and glial cells (Jacobson 1998). Moderate expression of A_3 receptor is found on cerebellum and hippocampus and lower expression in other areas of the brain (Fredholm et al., 2001). The A_3 receptor is coupled to inhibitory $G_{i\alpha 2}$-, $G_{i\alpha 3}$- and $G_{q/11}$ proteins that negatively impact on adenylyl cyclase activity (Palmer et al., 1995). Activation of A_3 receptors stimulates phospholipase C (PLC) signaling and increases levels of IP_3 and intracellular Ca^{2+}.

Less is known about the expression and function of A_3 receptors. Human expression of A_3 receptor has also been found on mast cells, suggesting a role in histamine release and inflammation (Ramkumar et al., 1993). In terms of neuroprotection, the A_3 receptor has been demonstrated to have a functional

relationship with A_1 receptor. Activation of rat A_3 receptors desensitizes A_1 receptor inhibition of excitatory transmission (Dunwiddie et al., 1997). A_3 receptor may have dual functions in neuroprotection and neurodegeneration. Administration of A_3 receptor agonist 2-chloro-N6-(3-iodobenzyl) adenosine-5-N-methyluronamide (IB–MECA) to gerbils significantly exacerbates the ischemic insult, although chronic stimulation in animals shows protection from stroke; conversely, the A_3 knockout mice show loss of neuroprotection (von Lubitz et al. 1999; Chen et al., 2006). In vitro studies have replicated this effect. Low nanomolar concentrations of the A_3 agonist IB–MECA affords neuroprotection to primary cortical cultures subjected to oxygen glucose deprivation (OGD) conditions (Chen et al., 2006). At higher micromolar concentrations (≥10 μM) apoptosis is induced through activation of caspases in astrocyte cultures (Appel et al., 2001). This is also seen in hippocampal neurons in culture (Von Lubitz et al., 1994).

2.0. Purines and Purinergic Signaling

2.1. Adenosine Triphosphate (ATP)

ATP has dual function as cellular source of energy and as an extracellular signaling molecule. ATP's effects on the heart and vasculature were first described by Drury and Szent-Györgyi in 1929. It is now known that ATP can be released as co-transmitter from nerves in both peripheral and central nervous systems (Burnstock, 1976, 2007, 2009). ATP displays typical characteristics of a classical neurotransmitter. Pre-synaptic ATP vesicular release can be detected in synaptic transmission and act on

post-synaptic receptors. ATP is stored in vesicles and is released or co-released with other neurotransmitters, including GABA, glutamate, noradrenaline and dopamine (Pankratov et al., 2006; Abbracchio et al., 2009). Also ATP can be released at the post-synaptic membrane and in response to other physiological states such as hypoxia (Zimmermann 1994). ATP can be hydrolyzed to ADP then AMP and ADO by membrane bound enzymes and act on purinergic receptors (P2).

Two subtypes of P2 receptors have been classified as ionotropic P2X and metabotropic P2Y families. Under physiological conditions, purinergic signaling is important in learning/memory, sleep/arousal, locomotor activity, mood/motivation and feeding behavior. Alternatively, pathological purinergic signaling is implicated in a variety of neurodegenerative diseases, psychiatric disorders, epilepsy, migraine, cognitive impairment and neuropathic pain (Burnstock et al., 2011).

2.2. Inosine

Inosine (INO) is a purine nucleoside and a primary metabolite of ADO. It was previously thought that products of ADO metabolism were nonreactive. However INO has been observed to have immunomodulatory and neuroprotective effects (Haskó et al., 2004). Like ADO, INO seems to be involved with extracellular signaling and involved with physiological and pathophysiological processes. INO can be formed from intracellular and extracellular pathways. In the first pathway, high levels of intracellular or extracellular ADO (associated with cellular stress from hypoxia or ischemia) are deaminated by ADO deaminase (ADA) to INO (Deussen, 2000). In the second pathway,

inosine monophosphate (IMP) is dephosphorylated by 5'nucleotidases to INO (Parkinson et al., 2006). Transport of INO from extracellular or intracellular space is mediated through both concentrative and equilibrative nucleoside transporters. ADO and INO are competitive substrates for these transporters (Pastor-Anglada et al. 2001).

ADO and INO are present at low concentrations and increase substantially under metabolically stressful conditions such as ischemia or inflammation (Ralevic and Burnstock 1998; Fredholm et al., 2001). Receptors for INO have not been identified; however micromolar concentrations of INO have been shown to activate A_{2A} and A_3 receptors (Fenster et al. 2000; Jin et al., 1997; Gomez and Sitkovsky 2003). The actions of INO are well documented in the immune system. INO plays a role in mast cell degranulation, suppresses pro-inflammatory agents, augments anti-inflammatory cytokines, and attenuates inflammatory and immune cell responses to ischemic-reperfusion injury (Haskó et al., 2000 and 2004).

The role of ADO as neuroprotectant during cerebral ischemia has been well established. Additionally, the mechanisms of ADO neuroprotection can be explained through the actions of ADO receptors as well as ADO metabolism. Other reports suggest that INO mediates ADO protective effects on the viability of rat astrocyte cell cultures in oxygen-glucose deprivation (OGD) conditions and this protection is reversed with the inhibition of intracellular adenosine deaminase (Haun et al., 1996) or inhibition of intracellular purine nucleoside phosphorylase (PNP) in astrocytes (Jurkowitz, et al. 1998; Litsky et al. (1999). As well, INO may promote axonal regrowth after injury in rats

(Benowitz et al. 1999). The therapeutic impact of INO still needs to be investigated. INO could potentially be a therapeutic agent in multiple sclerosis (Spitsin et al., 2001), Tourette's syndrome (Cheng et al., 1990) and cerebral ischemia.

2.3. Hypoxanthine

Hypoxanthine (HX) is a purine nucleobase and end product of extensive ATP catabolism. PNP converts INO to HX and ribose -1-phosphate. HX can be further metabolized by xanthine oxidase to xanthine. Alternatively HX can be converted to inosine monophosphate (IMP) by hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Purine metabolism in the CNS differs between species and organ systems. In human brain, levels of xanthine oxidase are low whereas HGPRT activity is high (Al-Khalidi and Chaglassian, 1965; Rosenbloom et al., 1967). During cerebral ischemia, HX levels are increased; although no neuroprotection is afforded by high levels of HX in the CNS. It has been suggested that HX accumulation in the brain could contribute to oxidative damage upon reoxygenation (White et al. 1984). Probably as a consequence of oxygen-glucose deprivation and utilization of energy rich purines, HX levels result from extensive purine metabolism (Hallgren et al., 1983).

2.4. Purinergic Receptors

2.4.1. P2X receptors

The P2X receptor subclass is a family of ligand-gated ion channel receptors. There are currently seven receptor subtypes, $P2X_{1-7}$, sharing 30-50% sequence homology. There are two trans-membrane (TM) spanning regions, TM1 involved with

channel gating and TM2 lines the channel pore. A large extracellular loop holds the ATP binding site. P2X receptors are permeable to Ca^{2+}, Na^{+} and K^{+} (Burnstock 2007b). The P2X receptors are composed of three subunits, either forming homomultimer or heteromultimer (Nicke et al., 1998; North 2002). There is much diversity among P2X receptors; there are six homomultimeric receptors $P2X_{1-5}$ and $P2X_{7}$. However the $P2X_{6}$ receptor does not evoke any currents but is functional in heteromultimer receptors (Burnstock 2007b). There are also six heteromultimeric P2X receptors $P2X_{2/3}$, $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{4/6}$ and $P2X_{1/4}$ (Roberts et al., 2006; Burnstock 2007b; Guo et al., 2007). In neuronal cells, all P2X subunits are expressed; the distribution of each receptor subtype remains to be fully characterized. Calcium permeability is variable among P2X receptors depending on subunit combinations. When P2X receptors are activated they can initiate cytosolic Ca^{2+} signals (Pankratov et al., 2008) and neurotransmitter release (Ferrari et al. 1999; Sperlágh et al. 2007) or mediate Ca^{2+} influx at resting membrane potentials in neurons (Pankratov et al., 2003). P2X receptors can interact with other receptors in the CNS such as GABA, NMDA and nicotinic ACh receptors (Khakh et al. 2000; Pankratov et al. 2008). P2X receptor activation mediates synaptic currents and is thought to be important in regulating synaptic plasticity of LTP or long-term depression (LTD). The role in these processes is still being investigated (Pankratov et al. 2008).

2.4.2. P2Y Receptors

The P2Y receptor family is G-protein coupled and have eight identifiable receptors, in which there are two distinct subtypes based on protein sequence and G-

protein coupling (Abbracchio et al. 2006). The first subgroup (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) primarily activates the G_q/G_{11} which then activates the PLC, IP_3 and intracellular Ca^{2+} signaling (Verkhratsky 2005; Burnstock 2007b). The second sub group (P2Y12, P2Y13, and P2Y14) is linked to the $G_{i/o}$, which reduces adenylyl cyclase levels and modulates ion channels (Abbracchio et al. 2006; Verkhratsky, 2005). Ligands of the P2Y receptor include adenine nucleotides (AN) ATP and ADP, uracil nucleotides (UTP and UDP), sugar nucleotides and recently identified cysteinyl-leukotrienes (Abbracchio et al., 2009). P2Y expression is prevalent throughout the CNS, in neurons and astrocytes (Parravicini et al. 2008; Ciana et al. 2006). Like the P2X receptor family, homo and heterodimers can be formed with other P2Y receptors (Ecke et al. 2008) or A_1 receptors (Fischer and Krügel 2007). P2Y receptor is involved with numerous molecular signaling pathways of tyrosine kinase receptors, integrins or nerve growth factor receptor (Erb et al., 2006; Arthur et al., 2005).

3.0 Adenosine Formation in the Brain

3.1 Intracellular Production and Metabolism of ADO

Possible sources of ADO can come from intracellular AN, cyclic adenosine monophosphate (cAMP) or S-adenosylhomocysteine (SAH). The main contribution to the formation of ADO occurs from the sequential dephosphorylation of ATP to AMP by cytosolic nucleotidases. Lesser pathways of ADO formation can occur by reversible conversion of SAH to adenosine and homocysteine by SAH hydrolase. Also ATP can

contribute to levels of cAMP by adenylyl cyclase; cAMP can be converted to AMP by phosphodiesterases (Latini et al, 1996).

Intracellular metabolism of ADO occurs from two enzymatic pathways. In the first pathway, ADO deaminase (ADA) irreversibly deaminates ADO to INO. In humans the deficiency of ADA activity correlates with immune dysfunction in severe combined immunodeficiency disease. The highest levels of human ADA are expressed in skin, lymph nodes, spleen, gastrointestinal tract and thymus while low levels of ADA are found in liver and kidney (Van der Weyden and Kelley 1976; Adams and Harkness 1976). In the CNS, highest levels are found in choroid plexus, hypothalamus, olfactory bulbs and superior colliculus; low levels are detected in the hippocampus and corpus callosum (Geiger and Nagy 1986). In vitro primary cell cultures of neurons and astrocytes also demonstrate ADA activity, however higher ADA activity occurs in astrocytes than neurons (Parkinson and Xiong, 2004; Ceballos et al,. 1994).

In humans, three forms of ADA have been found, soluble iso-enzymes (ADA_1 and ADA_2) and one ecto-enzyme (ecto-ADA) (Hirshhorn et al., 1980; Ratech et al., 1981b). Intracellular ADA activity is attributed to ADA_1, whereas ADA_2 is mainly found in plasma and serum (Ratech et al., 1981a). Ecto-ADA activity has been reported on the surface of hematopoietic cells and also in neurons (Kameota et al., 1993; Franco et al., 1986). Besides the obvious role in degrading ADO, ADA has other functions in regulating extracellular ADO and modulating A_1 receptor signaling, desensitization, and internalization (Ciruela et al., 1996; Saura et al. 1998).

The second pathway involves adenosine kinase (AK), which phosphorylates ADO, to AMP. AK preferentially utilizes ATP (as the source of phosphate) and yields AMP. The activity of AK requires magnesium (Mg^{2+}) and is most likely complexed to ATP ($MgATP^{2-}$) (Palella et al., 1980). Inhibition of this enzyme occurs when intracellular levels of free Mg^{2+} and ATP are low (Lindberg et al., 1967) or when ADO concentrations are high (Yamada et al., 1980). In regard with the latter, it has been suggested there are two binding sites on AK. The catalytic site has high affinity for ADO and the second regulatory site has lower affinity for ADO, which may be important for substrate inhibition of AK (Elalaoui et al., 1994). AK is conserved across species and has been found in a number of eukaryotic organisms. In humans, expression of AK is present in kidney, liver, spleen, placenta, pancreas and brain (Andres and Fox 1979; Snyder and Lukey 1982).

3.2 Extracellular Production and Metabolism of ADO

3.2.1 ATP release in the CNS

ATP has been long recognized as a source of energy for cells and, more recently, ATP has been described as a neurotransmitter of the CNS and PNS. On P2X receptors, ATP exerts an excitatory effect and extracellular degradation of ATP to ADO exhibits an inhibitory tone in the CNS (Edwards et al., 1992; Holton and Holton, 1954; Burnstock 1970; North 2002). In varying regions of the CNS, ATP is present in high concentrations, ranging from 2 to 4 mmol/kg (Kogure and Alonso 1978).

Vessicular neuronal ATP release was first described by Holton and Holton in 1953, through the electrical stimulation of axons. The mechanism of ATP release was

unclear, until it was described that ATP release was quantal (Burnstock and Holman, 1961; Pankratov et al., 2007) and present in synaptic vesicles at high concentrations (Sawynok et al., 1993; Bankston and Guidotti 1996). In agreement with these findings, the vesicular nucleotide transporter (VNUT) was identified by Sawada and colleagues in 2008. VNUT is a SLC17 anion transporter, which accumulates ATP and other substrates (ADP and GTP) into vesicles mediated by a Cl^- dependent process (Sawada et al., 2008). It is widely expressed throughout the brain; higher expression was noted in the cerebellar cortex, olfactory bulb and hippocampus. VNUT is also associated at excitatory and inhibitory nerve terminals on rat hippocampus (Larson et al., 2011). Following the uptake of ATP by VNUT, exocytosis mechanisms facilitate release into extracellular environment (Sawada et al., 2008; White 1978).

Vesicular ATP release has also been demonstrated in astrocytes (Coco et al., 2003; Zhang et al., 2007). As well, ATP is co-released with other neurotransmitters including ACh (Richardson and Brown, 1987), norepinephrine (Potter and White, 1980), GABA (Perez and Bruun, 1987) and glutamate (Mori et al., 2001). Other mechanisms have been noted, including ATP-binding cassette transporters, connexin or pannexin hemichannels, voltage-dependant anion channels, and $P2X_7$ receptors (Pankratov et al., 2006; Dubyak 2006; Scemes et al. 2007).

3.2.2 Extracellular enzymes

Generation of extracellular ADO can arise from extracellular dephosphorylation of AN by ecto-enzymes or ADO release from cells by transporters. The AN released into

the extracellular space are subject to metabolism by several ecto-enzyme families. The metabolism of AN is mediated by family of ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), ecto nucleoside pyrophosphatase and/or phosphodiesterase (e-NPP), alkaline phosphatases and ecto-5' nucleotidase (eN). These ecto-enzymes play an important role in purinergic signaling by controlling the availability of ligands of the P1, P2X and P2Y receptors.

3.2.2. i E-NTPDase

There are eight subtypes of E-NTPDases, only E-NTPDase 1-3 and 8 are membrane bound. E-NTPDase 1 (CD39, ecto-apyrase, ecto-ATP diphosphorylase) utilizes ATP or ADP as substrates; however ATP is directly dephosphorylated to AMP (Zimmerman 1996; Heine et al., 1999). E-NTPDase 1 can be found on microglia, endothelial and smooth muscle cells (Braun et al., 2000). In the CNS, high expression of E-NTPDase 1 is found in the thalamus, caudate putamen, and hippocampus (Langer et al., 2008). NTPDase 2 has selective preference for ATP and has been found on neuronal progenitor cells and non-myelinating Schwann cells (Braun et al., 2003, 2004). The activity of NTPDase 2 is mainly found in the dentate gyrus, subventricular zone and rostral migratory stream (Langer et al., 2008). NTPDase 3 mainly uses ATP and ADP as substrates. The ATPase activity of NTPDase 3 is mainly located in hypothalamus, hippocampus and cortex by immunohistochemistry (Langer et al., 2008). Subtypes NTPDase 5 and 6 is located in the intracellular compartment. These enzymes can be released extracelluarly and have specific ligand preference for nucleotide diphosphates.

Subtypes 4 and 7 are located intracellularly on the luminal side of the Golgi apparatus, lysosomal and autophagic vacuoles (Wang and Guidotti 1998; Biederbick et al. 1999).

3.2.2. ii E-NPPs

Currently there are seven variants of the e-NPP family with broad substrate specificity for hydrolyzing pyrophosphate and phosphodiester bonds. Three members of this family (e-NPP 1-3) are capable of nucleotide dephosphorylation of various substrates including ATP, ADP, nicotinamide adenine dinucleotide (NAD^+), and dinucleotide polyphosphates. In humans, expression of NPP 1-3 has been found in most tissues. In the CNS, NPP1 has been located in brain capillary endothelium but not in neurons or astrocytes (Harahap and Goding 1988). Expression of NPP2 in the CNS is limited to epithelial cells of the choroid plexus, cerebral spinal fluid (Narita et al., 1994) and in immature oligodendrocytes but not on neurons and astrocytes (Savaskan et al., 2007; Langer et al., 2008). NPP3 expression has been observed in the development of glial cells, where expression of NPP3 is seen in precursor cells but not in mature astrocytes (Rebbe and Hickman 1991; Blass-Kampmann et al., 1997).

3.2.2. iii Alkaline Phosphatase

Unlike the first two enzyme families, this enzyme family is the least studied of the ecto-nucleosidases. The alkaline phosphatases are widely distributed and have broad substrate specificity and can utilize nucleoside 5'- tri-, di-, monophosphates (Zimmermann 1996a). One alkaline phosphatase enzyme is capable of hydrolyzing ATP to ADO and releases inorganic pyrophosphate. They can be membrane bound as

glycosylphosphatidylinositol (GPI) anchored proteins or appear in serum. Other isoforms of alkaline phosphatases such as tissue-nonspecific alkaline phosphatase (TNAP), is widely expressed in the CNS including blood vessel endothelial cells, neuropil of olfactory bulb, cortex (Friede 1966; Fonta et al. 2004, 2005), hypothalmus, inferior /superior colliculi and dorsal / ventral medulla (Langer et al., 2008). The physiological role of TNAP is poorly understood. Although the optimal activity of the enzyme is at alkaline pH, it can also hydrolyze ATP and thus contribute to ADO production at physiological pH of 7.4. In a recent study, inhibition of TNAP by levamisole in mouse olfactory bulb demonstrated decreased neuron and astrocyte communication (Doengi et al., 2008). Thus TNAP may play a relevant role in purine nucleoside signaling.

3.2.2. iv Ecto 5' nucleotidase (eN)

The activity of eN is found in all tissues including the brain. In B and T lymphocytes, eN is also known as CD73 is a mark of maturation in these cells. In the CNS, eN is mainly located on the plasma membrane of astrocytes, microglia and oligodendrites (Schoen et al., 1987; Grondal et al., 1988). This enzyme has high affinity for AMP and can be competitively inhibited by ATP, ADP and α, β-methylene adenosine diphosphate (AOPCP) (Zimmerman 1996; Latini and Pedata 2001).

It is a GPI-anchored membrane protein and extracellular soluble forms can be produced from cleavage by phosphatidylinositol phospholipase C (Vogel et al., 1992). Extracellular soluble forms of eN have been noted in experimental conditions (Zimmermann 1992, 1996b). The enzyme appears in dimer form and requires zinc to

bind for functional activity (Knöfel and Sträter 1999). It utilizes nucleoside monophosphates and hydrolyzes to respective nucleosides and generates inorganic phosphate. Considering its direct conversion to nucleosides, eN plays a central role in generating ADO from extracellular released AN in the brain. In the CNS, eN has been described as a marker for myelin (Cammer and Tansey 1986; Kreutzberg 1978b), astrocytes and activated microglia (Kreutzberg, 1978 a, b, 1986; Gehrmann et al., 1991). In immature nerve cells, eN is transiently expressed during development and remodeling (Schoen and Kreutzberg 1994. 1995, Braun et al., 1995). In mature neurons the expression of eN is absent. In conjunction to enzymatic production of ADO from AN, eN also has a role in neuronal development. As well, under ischemic conditions, eN is up regulated in astrocytes and microglial cells as protective response to generate ADO which then limits the release of excitatory neurotransmitters which could further potentiate ischemic damage to the surrounding tissue (Braun et al., 1997).

3.2.3. Adenosine Transport

Extracellular and intracellular levels of ADO are regulated by its production and enzyme metabolizing processes. Nucleoside transporters facilitate transport of nucleosides across cell membranes to plasma or intracellular compartment. Two types of transporters have been identified: Equilibrative Nucleoside Transporter (ENT) and Concentrative Nucleoside Transporter (CNT).

3.2.3. i. Equilibrative Nucleoside Transporter (ENTs)

To date, four ENT subtypes have been cloned and identified in human, rat and mouse (Griffiths et al., 1997a, b; Baldwin et al., 2005; Barnes et al., 2006). ENTs demonstrate equilibrative and bi-directional transport of broad range of purines and pyrimidines. ENTs are most likely widely distributed in a variety of cell types and tissues (Griffiths et al., 1997a, b). Before ENT genes were identified, they were classified as *es* (equilibrative sensitive) or *ei* (equilibrative insensitive) to nitrobenzylthioinosine (or nitrobenzylmercaptopurine riboside, NBMPR) (Baldwin et al., 1999).

ENT1

In the classical nomenclature, ENT1 was identified as *es* and is also known as SLC29A1 (Young et al., 2008). ENT1 is inhibited by NBMPR at nanomolar concentrations and also by coronary vasodilator drugs dipyridamole (DPR) and dilazep (Young et al., 2008). ENT1 is also inhibited by tyrosine and serine/threonine kinase inhibitors (Huang et al., 2003) as well as lower potency benzodiazapines (Hammond et al., 1985). Human ENT1 (hENT1) has broad permeant range of purine and pyrimidine nucleosides but is unable to transport uridine (Griffiths et al. 1997 a, b; Ward et al. 2000). hENT1 may play a role in drug transport, as observed with anti-cancer nucleoside drugs (King et al., 2006) and rivavirin absorption and tissue distribution (Endres et al., 2009).

Expression of hENT1 is widespread on plasma membranes of many cell types and can also be found on nuclear (Mani et al., 1998) and mitochondrial membranes (Lee et al., 2006). The distribution of hENT1 has also been correlated with A_1 receptor

expression in human brain (Jennings et al., 2001). The highest protein level of hENT1 in the CNS has been found in the frontal and parietal cortex; intermediate levels in the thalamus, mid-brain, and basal ganglia; lowest expression was observed in the hippocampus and cerebellum (Jennings et al., 2001).

ENT2

Human ENT2 (hENT2; SLC29A2) (Young et al., 2008) are *ei* transporter; they are inhibited by NBMPR at micromolar concentrations. Inhibitors of hENT2 are the same as hENT1 (above) with some species differences in potencies. Similar to hENT1, hENT2 also has a broad range purine, pyrimidines selectivity and also transports hypoxanthine and other nucleobases (Griffiths et al., 1997 a, b; Yao et al., 2002). Pharmacology of antiviral nucleoside and anticancer nucleobase drugs are mediated through hENT2 (Yao et al., 2001; Nagai et al., 2003). Although hENT2 is co-expressed with hENT1, the activity of hENT2 may be less.

The pharmacology and physiology of ENT2 is not well known, mainly due to the fact that there are no selective pharmacologic agents to ENT2. ENT2 has been found in various types of tissues including heart, placenta, thymus, pancreas, prostate, kidney and is particularly abundant in skeletal tissue (Griffiths et al. 1997a; Crawford et al. 1998). In the CNS, hENT2 distribution was found in proteins isolated from the thalamus, medulla, midbrain, pons and cerebellum (Jennings et al., 2001).

ENT3

Human ENT3 (hENT3; SLC29A3) (Young et al., 2008) is not expressed on the plasma membrane because it is mainly localized to intracellular lysosomes (Pisoni and Thoene 1989; Baldwin et al., 2005) and mitochondria (Govindarajan et al., 2008). The protein sequence of ENT3 contains a long (51 residues) hydrophilic N-terminal region characteristic of endosomal/lysosomal targeting sequence. Removal of this sequence leads to localization of ENT3 to plasma membrane (Sandoval et al. 2000; Baldwin et al., 2005). Compared to hENT1, hENT3 is not affected by NBMPR or DPR or diltazem (Baldwin et al., 2005). The broad permeant selectivity of hENT3 includes purine and pyrimidine nucleosides including adenine, but hENT3 does not transport HX. In addition the hENT3 is capable of transporting chemotherapy and anti-cancer adenosine analogues such as cladribine (2-CdA, 2-chlorodeoxyadenosine) and fludarabine (2-fluoro-arabinosyladenine) (Baldwin et al., 2005). Expression of hENT3 has been found in the placenta in high abundance and is broadly distributed in the brain (Baldwin et al., 2005; Lu et al., 2004). Activity of hENT3 may be proton coupled; therefore functional activity requires acidic pH which mirrors the location of hENT3 in lysosomal compartments. The physiological function of ENT3 in lysosomes may contribute to recycling of nucleosides and nucleobases after nucleic acid breakdown (Pisoni and Thoene 1989).

ENT4

Human ENT4 (hENT4; SLC29A4) (Young et al., 2008) is the most genetically divergent of the ENTs. It is expressed on the plasma membrane and transports

adenosine at low affinity and monoamines in the brain and heart (Barnes et al., 2006; Zhou et al., 2007). Transport of nucleosides is activated at low pH which may indicate that hENT4 is coupled to proton exchange. ENT4 is not affected by micromolar concentrations of NBMPR, although it is partially inhibited by DPR and dilazep (Barnes et al., 2006). The ability of ENT4 to transport cancer drugs has yet to be determined, however in certain cancers, upregulation of ENT4 transcription which suggests its importance in cancer growth and survival (Li et al., 2008). In ischemia prone organs such as the brain and heart, ENT4 could regulate extracellular ADO levels under acidic conditions (Barnes et al., 2006). Expression of hENT4 mRNA is widespread in human tissues (Barnes et al. 2006). Within the CNS, localization of ENT4 was primarily to neuronal cell bodies and neuropil (Dahlin et al., 2007). Immunohistochemistry of mouse ENT4 in the CNS was observed in cerebral cortex, hippocampus, basal ganglia, cerebellum, thalamus and hypothalamus (Dahlin et al., 2007).

3.2.3. ii. Concentrative Nucleoside Transporters

The second nucleoside transporter family is the concentrative nucleoside transporters (CNT; also known in humans as SLC28) and composed of three members CNT1, CNT2 and CNT3 (King et al., 2006). Before the CNT designation the three subtypes of CNTs were previously identified as cit (CNT1), cif (CNT2) and cib (CNT3) (Griffith and Jarvis 1996; Cass 1995). In conjunction with ENTs, the CNTs also play a role in absorption, distribution and elimination of purines, pyrimidines and nucleoside drugs.

All CNTs are able to transport uridine and also have specific substrates within each subtype. The human CNT1 (hCNT1) is selective for pyrimidine nucleosides and can also bind to ADO at high affinity, although it is not translocated (Ritzel et al., 1997; Larrayoz et al. 2004). hCNT2 is selective for purine nucleosides (Ritzel et al., 1998) and hCNT3 is capable of transporting both purine and pyrimidine nucleosides (Ritzel et al., 2001; Wang et al. 1997; Smith et al. 2004). All three CNTs are able to transport anticancer and antiviral nucleoside drugs. As ENTs mediate the bidirectional translocation of purines, CNTs mediate unidirectional flow of purines and pyrimidines. This is an energy expending process that requires the facilitation of sodium ion exchange (Ritzel et al., 1998; Smith et al., 2004, 2005). The CNTs undergo conformational change that is Na^+ or H^+ dependent. This is followed by substrate binding and subsequent transfer of the substrate to the compartment (Smith et al., 2004, 2005). Altered conformational states have been found with different cation binding (Errasti-Murugarren et al., 2008). The ratio to which hCNT1/2 translocate substrates requires one sodium per nucleoside (1:1) (Ritzel et al. 1998; Smith et al. 2004), whereas hCNT3 requires two sodium ions per nucleoside (2:1) (Ritzel et al. 2001). The higher ratio suggests concentrative ability of moving permeants across cell membranes against concentration gradient (Ritzel et al., 2001; Smith et al., 2005, 2007). For hCNT3, the presence of protons the ratio changes to (1:1) (Smith et al., 2005, 2007). It is also reported that hCNT3 may have similar ratio as seen in hCNT1/2 (Larrayoz et al. 2004). In acidic environments with the presence of sodium, hCNT3 seem to have a select preference (high Km) for proton binding (Slugoski et al., 2009) which changes the

sodium: proton: nucleoside to 1:1:1 ratio [48]. These proton or sodium bound versions of hCNT3 changes substrate specificity at nucleoside binding pocket or translocation pore (Ritzel et al., 2001; Smith et al., 2005; Slugoski et al., 2008). Sodium coupled hCNT3 translocates purine, pyrimidine nucleosides, antiviral and anticancer nucleoside drugs. Proton-coupled hCNT3 is uridine selective and does not transport zidovudine (AZT, azidothymidine) (Smith et al., 2005).

The distribution of hCNTs is varied depending on subtype but all three CNTs are expressed in the brain. hCNT3 has broad distribution (Ritzel et al., 2001), where as hCNT1/2 are found in intracellular compartments (Mackey et al., 2005). Nucleoside transporters CNT and ENTs are expressed together in apical and basolateral kidney and intestinal epithelial cells, which may facilitate vectoral reabsorption of nucleosides and their derivatives (Mangravite et al. 2001, 2003; Mangravite and Giacomini 2003; Errasti-Murugarren et al. 2007). The distribution of CNT1 in rats showed significant levels in kidney and intestine but low levels in the CNS (Lu et al., 2004). Within the CNS, real-time polymerase chain reaction (RT-PCR) studies in the rat, showed CNT1 in high levels in brainstem and cortex; intermediate levels in choroid plexus, posterior hypothalamus, hippocampus, and cerebellum; and lowest expression in striatum and superior colliculus (Anderson et al., 1996). CNT2 was also found higher in kidney and intestine than brain (Lu et al., 2004). Transcripts of CNT2 were found in uniform distribution in the brain (Anderson et al., 1996) and high levels of rat CNT2 transcripts were found in neurons in select areas of the hippocampus and midbrain; intermediate levels of CNT2 were found in neurons of basal ganglia, cortex, hypothalamus and granule area of cerebellum

(Guillen-Gomez et al., 2004). In vitro cultures of mouse neurons and astrocytes reported CNT2 in neurons and astrocytes (Nagai et al., 2005; Peng et al., 2005). In mouse (Lu et al., 2004) and human (Ritzel et al., 2005), CNT3 has been reported at low levels throughout the brain; however, in rat neuron and astrocyte cultures CNT3 levels were not detectable (Nagai et al., 2005).

4.0. Purinergic Signaling: Cell to Cell Communication

Neurons are the basic unit to which information is processed in the CNS. The development of electrical stimulation and recording devices has yielded a lot of information about communication between neurons, as they propagate fast electrical signals or action potentials.

Glial cells of the brain consist of two classes macroglia, comprise of astrocytes and oligodendrocytes and microglia, which are the resident macrophages in the CNS. Astrocytes provide metabolic support to neurons such as scavenging neurotransmitters released at the synapse; regulate ion and water homeostasis; release neurotrophic factors; remove metabolites and waste products; and provide structural support at the blood-brain barrier (Chen and Swanson 2003). The notion that astrocytes were "non-excitable" support cells has changed in the last 25 years. Astrocytes are not electrically excited but do exhibit intracellular Ca^{2+} signaling in response to neuronal activity by expressing a variety of neurotransmitter receptors and ion channels (Verkhratsky and Kettenmann 1996; Vernadakis 1996).

Astrocytes also respond to a variety of transmitters in the CNS, such as glutamate, norepinephrine, histamine, ACh, ATP and GABA (Shelton and McCarthy 2000; Kulik et al., 1999; Duffy and MacVicar 1995; Wang et al., 2000; Guthrie et al., 1999; Cotrina, et al 1998; Haydon 2001). There are two types of Ca^{2+} signaling: Ca^{2+} oscillation and Ca^{2+} waves (Cotrina and Nedergaard 2005). Calcium oscillations are repeated monophasic increases of intracellular Ca^{2+} triggered by exposure to glutamate, GABA and ATP. Ca^{2+} oscillations are typically limited to within the cell and involve activation of PLA_2, IP_3 and Ca^{2+} release from intracellular stores (Berridge et al., 2003). Long range signaling is propagated by Ca^{2+} waves; generated by electrical stimulation, mechanical stimulation, glutamate, and ATP (Wang et al., 2006). Calcium waves are propagated at a velocity of 8-20 micrometers per second (Haydon 2001; Takana et al., 2009). The Ca^{2+} wave modulating astrocyte-astrocyte signaling is mediated by ATP and P2Y receptors (Cortina et al., 1998, 2000; Takano et al., 2009). Several mechanisms of ATP release could come from channel mediated release, exocytosis, connexin hemichannels or pannexins (Haydon 2006). As astrocytes are integrated with neuronal synapses, they have the potential to modulate synaptic transmission. For instance, Ca^{2+} oscillations in astrocytes can initiate neuronal excitation through the release of glutamate and/or D-serine and then evoke NMDA-receptor currents (Parri et al., 2001). As well, astrocytes have been demonstrated to potentiate synaptic inhibition in the hippocampus by $GABA_B$ receptor-dependent pathway (Kang et al., 1998). From these experiments, glutamate and ATP/adenosine can mediate astrocyte-neuron signaling (Nedergaard et al., 2002).

5.0. The Role of Adenosine in Physiological Conditions

ADO is produced from ATP during conditions of energy depletion and has been described as a "retaliatory metabolite" (Newby 1984). When ADO is released extracellularly under these conditions, it acts on A_1 receptors to decrease cell activity while acting on A_2 receptors to increase cerebral blood flow to deliver oxygen and glucose. Therefore decreasing the energy demands of cells will shift toward ATP forming processes.

This view is challenged by the actions of ADO within the CNS at physiological conditions. In normal physiology, ADO and its receptors participate in sleep/awake cycles, influence neuronal excitability and regulate cerebral blood flow. Physiological stimuli can increase levels of ADO through increases in temperature (Gabriel et al., 1998; Masino & Dunwiddie 1999). In addition, caffeine the most widely consumed drug in humans is normally consumed for it stimulant properties but is a non-selective ADO receptor antagonist. Furthermore different cell types in the CNS can release AN, which can act on P2 receptors or become dephosphorylated by extracellular nucleotidases to ADO. The extracellular ADO and can then act on ADO receptors or be moved into cells by nucleoside transporters.

5.1. Regulation of Sleep and Awake Cycles

ADO plays a role in energy homeostasis and plays a role in promoting or maintaining sleep and arousal. In animals, the function of sleep is to replenish energy stores but also it consolidates information to memory in organisms with well-developed

nervous systems. Neuronal activity in the brain requires constant supply of energy either from glucose or to a smaller extent, glycogen from astrocytic stores. As neuron activity increases so does energy (ATP) consumption and resulting in the end-product ADO. Sleep then replenishes energy rich compounds in the brain (Dworak et al., 2007; Magistretti 2009).

ADO in the basal forebrain appears to regulate sleep and wakefulness. The first direct microdialysis measurements *in vivo* of ADO in the CNS were described in the sleep-awake cycles in cats (Porkka-Heiskanen et al 1997, 2000). ADO levels in the forebrain increased during prolonged wakefulness and then decreased during sleep. The metabolic enzymes of ADO formation or degradation exhibit diurnal variations. The enzymatic activities of AK, cytosolic and ecto 5' nucleotidases were low in the cortex and forebrain during the rest phase, indicating decreased energy metabolism (Alanko et al., 2003). During wakefulness AK and cytosolic 5'nucleotidases were elevated (Mackiewicz et al., 2003).

The effect of caffeine promotes wakefulness by antagonizing ADO receptors. The ADO-mediated sleep/awake cycles in the CNS are attributed to the A_1 and A_{2A} receptors. ADO receptor antagonists promote wakefulness (Lin et al., 1997); whereas ADO agonists promote sleep (Portas et al., 1997). Stimulation of the A_1 receptors increased total sleep or deep sleep by direct application of ADO or A_1 agonist N^6-cyclopentyladenosine (CPA) suppressed rapid eye movement (REM) sleep while increasing slow-wave sleep (SWS) in nonREM sleep (Benington et al., 1995; Schwierin et

al., 1996). Conversely A_1 antagonist 8-cyclopentyltheophylline (CPT) promotes wakefulness by decreasing SWS in REM sleep (Strecker et al., 2000). A_1 receptor mediated sedation induced by ethanol may be mediated by inhibition of the wake-promoting neurons in the basal forebrain. A_1 mediated inhibition of basal forebrain neurons maybe responsible for the sedative effects of ethanol; correspondingly, wakefulness is increased during ethanol withdrawal as well as reduced A_1 receptor and ENT1 expression (Thakkar et al., 2010; Sharma et al., 2010). Other evidence suggests that A_{2A} receptors may be involved. The A_{2A} agonist CGS21680 promotes REM and nonREM sleep.

6.0. The Role of Adenosine in Pathophysiological Conditions

6.1. Adenosine in Neuroprotection

Extracellular levels of ADO can arise under pathophysiological events such as ischemia/hypoxia and seizure activity. Pathophysiological stimuli greatly enhance extracellular levels of ADO. Activation of the A_1 receptors effectively inhibits the release of neurotransmitters and hyperpolarizes neurons. In these circumstances, neuroprotection by ADO reduces damage to neuronal tissue. The application of ADO receptor antagonists exacerbates damage (Mitchell et al., 1995).

A brief period of hypoxia or ischemia reduces tissue damage in the brain and heart after subsequent ischemic insult (Miura and Tsuchida 1999). Neuroprotection from pre-conditioning of excitable tissues is mediated by the actions of A_1 and A_3 receptors (Stambaugh et al 1997, Liang & Jacobson1998).

6.1.1. Ischemic/Hypoxic Conditions

The energy and metabolic requirements of the brain account for 20% of oxygen consumption and 25% glucose utilization (Sokoloff et al., 1977; Kety, 1957). Ischemic stroke occurs when there is an interruption of blood flow in the brain. Thus energy requirements cannot be maintained resulting in altered neuronal function and eventual cell death (Dirnagl et al., 1999). Ischemic stroke is the third leading cause of death in Canadians. About $2.7 billion dollars are spent due to physician services, hospital cost, loss in wages and productivity. Currently tissue plasminogen -activator (tPA) is the only drug approved for use in stroke patients and even this drug is limited to only a few patients, based on the time from stroke onset and numerous patient factors. The pharmacological action of tPA is to activate plasmin to degrade blood clots and restore blood flow to ischemic regions.

At the cellular level, ischemia causes damage to neurons through consumption of oxygen and glucose leading to ATP depletion. Numerous processes for survival are energy-dependent. Ion gradients, usually maintained by ATP, result in the loss of K^+ currents and influx of sodium, chloride and calcium ions. The substantial increase in intracellular Ca^{2+} can activate a whole host of enzymatic processes and cell signaling cascades which ultimately lead to apoptosis or necrosis (Orrenius et al., 2003; Penn and Loewenstein 1966; Simon et al., 1984). The loss of ion gradients is also accompanied by cellular edema in neurons and glia; generation of reactive oxygen species; excessive neuronal depolarization and reduced uptake and enhanced release of excitatory neurotransmitters (Dirnagl et al., 1999; Karaszewski et al., 2009, Deb et al., 2010).

Glutamate is the main excitatory neurotransmitter in the CNS and has been described to have toxic effects on neurons (Olney 1969). Glutamate activates ionotropic N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-propionate (AMPA) and kainate receptors, all of which mediate cation influx.

The endogenous levels of ADO increase in hypoxic and ischemic conditions (Hagberg et al. 1987; Matsumoto et al. 1992; Latini and Pedata 2001; Rudolphi et al., 1992b). The neuroprotection by ADO decreases neuronal excitability; the actions are attributed to the A_1 receptor, which 1) inhibits glutamate transmitter release (de Mendoncą et al., 2000; Fredholm 1996; Rudolphi et al., 1992a); 2) hyperpolarizes neurons (Dunwiddie and Masino 2001); and 3) limits Ca^{2+} entry by inhibition of Ca^{2+} channels (Fredholm 1996; Greene and Hass 1991). In contrast, activation of the A_{2A} receptor exacerbates excitotoxicity by the release of glutamate and aspartate (Popoli et al., 1997; Gui et al., 1999).

Current research in this field is actively investigating stimuli that increase ADO concentrations and the contribution of intracellular or extracellular pathways of ADO formation under hypoxic and ischemic conditions. The activation of NMDA receptors also increases extracellular ADO levels in experimental conditions. The extracellular ADO levels can also be manipulated *in vitro* through nucleoside transport and eN inhibitors. Under *in vivo* conditions, extracellular formation of ADO was observed with eN inhibition in basal and NMDA-stimulated conditions resulting in decreased ADO levels (MacDonald and White 1985; Hoehn and White 1990; Craig and White 1993). As well

with nucleoside transporter inhibitor, ADO levels were increased preventing reuptake of ADO (Delaney and Geiger 1998). Other studies report the opposite: *in vitro* experiments with neurons and astrocytes or slice preparations suggest adenosine release from intracellular forming pathways (Meghji et al., 1989; Lloyd et al., 1993).

6.1.2. Epilepsy

Epilepsy is characterized as a neurological disorder with recurring seizures. Mechanisms that modulate neuronal excitability and inhibition are in a state of imbalance (Löscher and Köhling 2010). In addition to inhibitory neuromodulatory effects, ADO also exhibits antiepileptic effects and functions as an "endogenous anticonvulsant" (Dragunow 1988). At excitatory synapses A_1 receptors are abundant. Activation of A_1 receptors decreases glutamate release and hyperpolarizes neurons and, similar to conditions like ischemia, endogenous levels of ADO increase during seizure activity (During and Spencer 1992; Boison 2008).

The anticonvulsant effects of ADO have been demonstrated in numerous experimental models. Administration of ADO agonists showed reduced epileptic activity mediated through the activation of A_1 receptors (Dunwiddie and Worth 1982; Barraco et al., 1984; Zhang et al., 1990). Although ADO agonists have demonstrated reduced epileptic activity, clinically they are not used due to undesirable peripheral effects – decreased blood pressure, heart rate and temperature (Dunwiddie 1999).

6.2. Adenosine and Neurodegeration

6.2.1. Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder of muscle movement characterized by the loss of dopaminergic neurons in the nigrostriatal neurons. Symptoms of PD include tremors, muscular rigidity, bradykinesia, postural and gait abnormalities. The etiology of PD is unknown, but may be due to genetic or environmental factors. The current pharmacological treatment for PD is levodopa, (L-dopa) the precursor to dopamine, which provides symptomatic relief but does not reverse neuronal degeneration. Degeneration of dopaminergic neurons decreases dopamine levels, which creates an imbalance of inhibitory dopamine to excitatory ACh levels in the striatum. Levodopa aims to increase levels of dopamine. After crossing the blood brain barrier, levodopa is metabolized to dopamine in the brain. Carbidopa is a dopa decarboxylase inhibitor and is combined with L-dopa to reduce peripheral and GI metabolism of L-dopa thereby increasing L-dopa levels in the brain.

New therapeutics for PD is directed at the antagonism of A_{2A} receptors and show great promise. In humans, co-localized A_{2A} receptors and D_2 receptors were demonstrated and shown to inhibit reciprocal antagonistic interactions (Diaz-Cabiale et al., 2001). As well in rats, parkinsonian-like muscle rigidity produced by reserpine or haloperidol was antagonized by selective A_{2A} receptor antagonist SCH 58261 and L-dopa. The combined administration of SCH 58261 and L-dopa produced a synergistic effect to decrease parkinsonian-like muscle rigidity in rats (Wardas et al., 2001). In a similar manner to carbidopa, the co-administration of L-dopa and A_{2A} antagonist will

allow lower doses of L-dopa, minimizing side effects and tachyphlaxis. In non-human primate models of PD, administration of selective A_{2A} antagonist KW-6002 also potentiated the effects of L-dopa with limited dyskinetic movements (Kanda et al., 1998; Grondin et al., 1999). These results were also reflected in human phase II clinical trials with A_{2A} antagonist KW-6002 and L-dopa improved symptoms with minimal side effects. The use of KW-6002 was determined to be safe and well tolerated in PD patients without increasing dyskinesia (LeWitt et al., 2008).

Caffeine is non-selective ADO receptor antagonist and consumption of coffee has been associated with reduced risk of PD. In support of this, caffeine given to rats, at doses comparable to human consumption, protected the loss of striatal dopamine neurons in the neurotoxin MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine)-induced mouse model of PD (Chen et al., 2001). The potential for disease modification by caffeine initiated several progressive longitudinal studies. In a large, long term prospective study 8000 Japanese-American men in Honolulu Heart Program were followed for 30 years. The risk of PD in non-drinkers vs. heavy coffee drinkers was five times higher (Ross et al., 2000). Three other studies were conducted in ethnically diverse large cohorts, involving the Health Professionals' Follow-Up Study and the Nurses' Health Study (Ascherio et al. 2001) as well as the Finnish Mobile Clinic Health Examination Survey (Saaksjarvi et al. 2007). These studies confirmed the relationship between coffee consumption and decreased risk of PD in men.

6.3. Adenosine and Mood Disorders

Dopamine and glutamate are the main neurotransmitter systems responsible for behavior, mood and cognition. In addition, the regulation of sleep, locomotion, anxiety, cognition and memory are regulated by ADO and its receptors (A_1 and A_{2A}) (Dunwiddie and Masino 2001; Fredholm et al., 2005). ADO's interaction with dopaminergic and glutaminergic systems implies it has a role in behavior and cognitive function. Alterations in ADO signaling could give rise to psychiatric symptoms in anxiety/depression and schizophrenia (Ribero et al., 2003).

The basis of this interaction is mediated by the A_{2A} receptor. Activation of A_{2A} receptors regulates neuronal excitability and synaptic plasticity through the release of ACh, glutamate and dopamine, but inhibits gamma aminobutyric acid (GABA) neurotransmission (Kirk and Richardson 1995; Cunha and Ribeiro 2000; Ciruela et al., 2006). In addition A_{2A} receptor has been shown to co-localize with A_1 receptor and other receptor types such as dopamine D_2 receptor, cannabinoid CB_1 receptor, metabotropic glutamate receptor subtype 5 (mGluR5) and nicotinic ACh receptor (Dunwiddie and Masino 2001, Ferre et al., 1991, 2002).

6.3.1. Anxiety and Depression

Anxiety has often been associated with caffeine or ethanol consumption. Caffeine is a global antagonist to ADO receptors and ethanol inhibits nucleoside transporter re- uptake of ADO (Clark and Dar 1989; Nagy et al., 1990; Krauss et al., 1993). Some of the behavioral effects of caffeine and ethanol are attributed to the A_1

and A_{2A} receptors. Also anxiogenic effects have been observed with A_{2A} receptor agonist. In A_{2A} knockout mice, anxiety-related behaviors were enhanced in relation to wild type mice (Ledent et al., 1997; Berrendero et al., 2003 Bilbao et al., 2006). Administration of ADO was shown to be anxiolytic whereas treatment with caffeine or theophylline induced anxiogenic behaviors at high doses. The anxiolytic effects of ADO were reversed with pretreatment of caffeine or theophylline (Kulkarni et al., 2007). The role of A_1 or A_{2A} receptors in anxiety still has yet to be defined.

Depression is a debilitating condition with emotional, cognitive, somatic and psychomotor deficits. Several neurotransmitter systems are implicated in depression such as dopaminergic, serotoninergic and corticotrophin systems (Cunha et al., 2008). There is evidence to suggest a role of ADO in modulating depression. Nortriptiline, chlorimipramine and desipramine are classical tricyclic antidepressants that have binding activity to ADO receptors and show dose-dependent reductions of ecto-nucleotidase activity (Deckert and Gleiter 1989; Barcellos et al., 1998). Administration of ADO in the periphery orby intra-cerebroventricular injection showed anti-depressant effects, while also activating opioid receptors and nitric oxide-cGMP system (Kaster et al., 2004, 2005, 2007). Conversely, other studies have shown that ADO showed depressant-like effects in behavioral studies (Minor et al., 1994; Woodson et al., 1998; Hunter et al., 2003). Clearly the role of ADO in depression has yet to be defined. However, antagonism of the A_{2A} receptor has shown anti-depressant effects. In animal models of depression, tail suspension and forced swim tests, A_{2A} receptor antagonists reversed “behavioral despair”. In addition, the D_2 antagonist, haloperidol reversed the

effects of A_{2A} antagonism (El Yacoubi et al., 2001, 2003). This would suggest that blockade of A_{2A} receptor restored dopaminergic signaling and alleviated symptoms of depression. Thus a novel therapeutic target in depression could be through the modulation of A_{2A} receptors.

6.3.2. Schizophrenia

Schizophrenia is a complex psychiatric disorder associated with cognitive and behavioral dysfunctions. Clinically, schizophrenic symptoms are divided into i) positive symptoms characterized by delusions, hallucinations, and disorganized thinking ii) negative symptoms including anhedonia, blunted effect and social withdrawal iii) affective symptoms such as depression or mania and iv) cognitive deficits indicated by poor memory and attention (Ross et al., 2006). The neurochemical basis for schizophrenia has not been determined although several models associate with the dopaminergic, glutaminergic, serotonergic, cholinergic, or GABAergic neurotransmitter systems (Lara and Souza 2000). Current pharmacological therapy is based on "dopamine-hyper-function" and "glutamate hypo-function" hypothesis of schizophrenia. The positive symptoms are attributed to excessive dopamine release and drugs that antagonizes the dopamine D_2 receptor, although the effectiveness is limited by serious side effects. The negative and cognitive symptoms in schizophrenia are not treated by the blockade of the D_2 receptor. Treatment of these symptoms is thought to be increasing glutamate and NMDA receptor activity. However excessive stimulation of the NMDA receptor may result in excitotoxicity (Citri and Malenka 2008; Malenka and Nicoll 1993).

The neuromodulatory functions of ADO on neurotransmitter release has led to the ADO-hypofunction hypothesis which states that dysfunctions in the purinergic signaling pathway lead to decreased adenosinergic tone in the brain which leads to the imbalance of dopamine and glutamate neurotransmission which account for wide range of symptoms in schizophrenia (Lara and Souza 2000, 2006). For instance, transgenic mice with over expression of AK have reduced ADO levels in the brain and subsequent down regulation of the A_1 and A_{2A} receptors. These mice exhibited transient increases in locomotor activity in novel situations and this activity declined to control or below levels, demonstrating locomotor habituation. Also, the mice showed pronounced learning deficits in Morris water maze and in Pavlovian conditioning. The mice were exposed to psychostimulants such as amphetamine and exhibited reduced activity, where as NMDA receptor antagonist MK-801 showed increased locomotor reaction (Yee et al., 2007).

In schizophrenic patients, caffeine seems to exacerbate symptoms (De Freitas and Schwartz 1979; Lucas et al., 1990; Mayo et al., 1993; Nickell and Uhde 1994), where as the nucleoside transport inhibitiors DPR or the xanthine oxidase inhibitor allopurinol may offer symptomatic relief (Akhondzadeh et al., 2000, 2005).

The A_{2A} receptor in schizophrenia is coupled to D_2 receptors in the striatum. A_{2A} receptor agonist appears to act as a D_2 antagonist by reducing its function and affinity for dopamine. Also caffeine has been shown to substantially increase dopamine which aggravates symptoms of a schizophrenic patient (Powell et al., 2001). These data suggest that activation of A_{2A} receptors could reduce dopamine levels in the striatum.

Alternatively, the hypoactivity of the NMDA receptor could explain the negative symptoms of schizophrenia (Olney and Farber 1995; Coyle and Tsai 2004; Farber 2003). Inhibition of NMDA receptors is known to impair LTP in animals (Davis et al., 1992, Morris, 1989 and Morris et al., 1986). In humans NMDA receptor blockade can give rise to impulsive and psychotic-like behavior from psychomimetics phencyclidine (PCP) and ketamine (Tonkiss et al., 1988; Farber 2003). Conversely, co-agonists of the NMDA receptor, D-serine and glycine have been shown to improve the negative symptoms in schizophrenia (Coyle and Tsai 2004; Javitt 2008). The ADO receptors A_1 and A_{2A} exhibit different modulation profiles of NMDA receptor in the striatum. The A_1 inhibits excitatory neurotransmission by decreasing glutamate release; A_1 antagonists could potentiate glutamate activity and improve cognition (de Mendonca et al., 1995; Dunwiddie and Masino 2001; Takahashi et al., 2008). However, the psychostimulant effects from NMDA receptor antagonism could be abolished by A_{2A} receptor antagonism in the striatum (Gerevich et al., 1992; Kafka and Corbett 1996; Popoli et al., 1998; Wardas et al., 2001).

6.4. Adenosine and Drugs of Abuse

Caffeine is widely consumed for its psychostimulant effects and yet not considered by the DSM-IV as a drug of abuse. Mostly consumed for its stimulatory effects, caffeine acts as a non-selective ADO receptor antagonist. Caffeine could be described as a drug of abuse because caffeine consumption is habitual, and produces tolerance, dependence and withdrawal syndrome after stopping consumption. On the

other hand, drugs of abuse such as ethanol and opiates could be connected to ADO as well.

6.4.1. Ethanol

Ethanol has been thought to be involved with alterations in ADO metabolism, uptake and receptor activity. The effects of ethanol can be attributed to increased levels of ADO in the brain. Ethanol metabolism results in substantial concentrations of acetate and increased AMP levels which is then metabolized to ADO (Carmichael et al., 1991). Ethanol has been reported to inhibit ENT1 (Nagy et al., 1990) and the elevated ADO levels contribute to the behavioral effects of ethanol. Ethanol-induced sedation and ataxia are attributed to A_1 receptor mediated effects. In rodents, these effects are ameliorated if given A_1 antagonist (Procter et al., 1985; Barista et al., 2005; Thakkar et al., 2010).

6.4.2. Opiates

Drug dependence to opiates or stimulants (ex cocaine or heroin) has been associated with alterations in LTP, the basis of learning and memory in the brain (Bliss and Collingridge 1993). Chronic use of opiates or heroin leads to impairment of LTP (Pu et al., 2002; Salmanzadeh et al., 2003; Bao et al., 2007) contributing to cognitive deficits (Cipolli and Galliani, 1987; Guerra et al., 1987; Spain and Newsom, 1991) associated with opiate or drug abuse.

Opiates have been shown to increase release of ADO in the brain spinal cord and peripheral nervous system (Fredholm & Vernet 1978, Stone 1981, Cahill et al 1996;

Sweeny et al., 1993). The actions of ADO and ADO agonists also inhibit hippocampal LTP through the A_1 receptor (Arai et al., 1990; de Mendonça and Ribeiro, 1990; Alzheimer et al., 1991; Forghani and Krnjevic, 1995). Activation of the pleasure and reward centers, the nucleus accumbens and ventral tegmental region, showed increased A_1 receptor activation (Bonci and Williams, 1996; Shoji et al., 1999; Fiorillo and Williams, 2000). Recently it was determined that the A_1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) was able to reverse LTP impairment of hippocampal CA1 and improve spatial memory in chronic morphine-induced mice (Lu et al. 2010).

Summary

Overall, ADO is a neuromodulator, affecting neuronal synaptic transmission in an excitatory or inhibitory manner. The diverse effects of ADO on the CNS are dependent on the brain region and ADO receptor subtype. The regulation of ADO is based on activities of metabolizing enzymes and transporters in the intra and extracellular pathways. Neuroprotection by ADO imparts an inhibitory tone on the CNS especially in pathophysiological conditions such as ischemia or seizures. ADO is integrated with other neurotransmitter systems. Irregularities in ADO concentrations or altered receptor signaling may give rise to characteristic pathologies.

A variety of methods used to understand the formation/release of ADO may give different conclusions based on the preparations used and stimulus to evoke ADO release. Therefore further clarification on ADO production in release mechanisms in response to pathological stimuli is required.

Chapter 2: Objectives and Rationale

Sudden increases in endogenous ADO occur in response to cerebral ischemia. The regulation of ADO levels in these conditions has yet to be defined by cellular sources and by the pathways of ADO formation. This research further investigated the roles of neurons and astrocytes as sources of ADO in ischemic/hypoxic conditions, as well, the contributions of intra- and extracellular pathways of ADO formation. We used genetically modified mice to tease out the cellular contributions and the enzymes/transporters involved with production of ADO in ischemic-like conditions. Two mouse models were utilized; the transgenic hENT1 with neuronal specific expression and the knockout CD73 with decreased expression of eN in astrocytes. We modeled conditions of ischemia using NMDA, an agonist for the NMDA glutamate receptor on neurons, whereas in astrocytes glucose or oxygen-glucose deprivation was used to evoke significant ADO release. Previous research in this laboratory has shown that under ATP-depleting conditions neurons directly release ADO whereas astrocytes release AN, which is then metabolized to ADO (Parkinson et al., 2002).

ADO levels in the brain are under control of transporters and enzymes in the ADO forming pathways. ENT1 and ENT2 transporters facilitate the movement of nucleosides, including adenosine, across cell membranes. Both ENT1 and ENT2 transporters can be inhibited pharmacologically by DPR at micromolar concentrations. ENT1 can be inhibited selectively with NBMPR at nanomolar concentrations. ENT knockout mice exhibited decreased ADO levels in the brain, and yet increased levels in peripheral regions (Choi et al., 2004; Rose et al., 2011). Also these mice exhibited ischemic cardioprotection, presumably from increased A_1 receptor activation (Rose et

al., 2010). Therefore, transgenic mice with hENT1 expressed under the control of a rat promoter for neuron specific enolase were used to evaluate the role of neuronal ENTs in the regulation of adenosine levels. The enzyme eN or CD73 is primarily expressed in astrocytes and is an important enzyme for the extracellular formation of ADO. The presence of ATP, ADP or AOPCP inhibits eN at micromolar concentrations. In rat co-cultures, stimulated with NMDA, ADO levels were decreased in the presence of AOPCP [reference]. Therefore, CD73 knockout mice were used to study the importance of eN in the regulation of adenosine levels during excitotoxic conditions.

Hypothesis 1: ENTs are bi-directional transporters mediating uptake or release. The influx or efflux is dependent on the concentration gradients of ADO or INO. I hypothesize that hENT1 transgenic neurons release greater quantities of purines than wild type neurons in response to NMDA.

Hypothesis 2: The expression eN is primarily found on astrocytes, facilitating the metabolism of extracellular AMP to ADO. I hypothesize that astrocytes from CD73 KO mice produce less extracellular ADO than wild type astrocytes in basal conditions and in response to glucose and oxygen-glucose depletion.

Hypothesis 3: Previous studies have indicated that eN is important for ADO formation in brain slices and in co-cultures of neurons and astrocytes. I hypothesize that co-cultures containing astrocytes from CD73 KO mice will show less ADO production than co-cultures containing wild type astrocytes in response to treatment with NMDA, an experimental model of ischemic excitotoxicity.

Chapter 3: Experimental Methods

3.1 Materials

Neurobasal media, Dulbecco's modified Eagle medium –F12 (DMEM-F12), B-27 supplement, fetal bovine serum (FBS), L-glutamine, and antibiotic/antimycotic (penicillin, streptomycin, amphotericin B) were purchased from Invitrogen (Burlington, Ontario, Canada). [^{3}H]Adenine was purchased from Perkin Elmer (Boston, MA). [^{14}C]AMP was purchased from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). Silica gel-coated glass plates were obtained from Fisher Scientific (Whitby, Ontario, Canada). Dipyridamole (DPR), α, β-methylene ADP (AOPCP), NBMPR, N-methyl-D-aspartate (NMDA), glutamic acid, 2-deoxyglucose (2DG), and adenosine monophosphate (AMP) were purchased from Sigma-Aldrich Canada (Oakville, ON).

3.2 Mice

Transgenic mice on a CD1 background, expressing the transgene containing a rat promoter region for neuron specific enolase was coupled to the coding sequence of human equilibrative nucleoside transporter 1 (hENT1) (Parkinson et al., 2009).. To obtain neuron cultures enriched in hENT1 expression, either heterozygous (Tg/+) mice were mated or heterozygous was mated to homozygous (Tg/Tg). As controls, CD1 neuron cultures were obtained from timed pregnant CD1 mice obtained from the animal facility.

CD73 knockout mice were obtained from Dr. Linda Thompson (Thompson et al., 2004) and bred locally. Wt C57Bl6 and CD73 KO pups were used at 0-3 days. All procedures with animals were in accordance with animal care guidelines set by the

Canadian Council on Animal Care approved by the University of Manitoba Animal Protocol Management and Review Committee.

3.3 Cell Culture

The cerebral cortices from gestational day 17 CD1 or hENT1 mice were isolated from whole brain for primary neuron cultures. Cortices were dissociated with sterilized disposable pipette tips (1ml) and glass pipette. The cells were incubated for 1 hour at 37^{o}C in 150-cm^{2} flasks to reduce astrocyte growth in the neuron preparation. After incubation, cells were further dissociated with 21-gauge needle and 10-ml syringe. Cells were plated on poly-D lysine coated 12-well plates. Neurons were plated in 0.5 ml Neurobasal media containing 2% B-27 supplement, 1x antibiotic/antimycotic (100 units/mL of penicillin, 100 units/mL of streptomycin, and 0.25 units/mL of amphotericin B), 500 µM L-glutamine, and 25 µM glutamic acid. After 4 days *in vitro* (DIV), half the media was replaced with fresh Neurobasal (without glutamic acid) and following this the media was changed in this manner every 7 days. Experiments were performed 24 hours after the second media change on 11 DIV.

Primary astrocytes were cultured from cerebral cortices from C57Bl6 or CD73 knockout pups (0-3 days). Once isolated, cortices were triturated several times with sterilized disposable pipette tips (1ml) and glass pipettes. Cells were then centrifuged at 1500 rpm for 5 min and plated on T-150 flasks. After 5-7 DIV, flasks were shaken at 300 rpm for 14 hr to remove microglia. Astrocytes were fed every three days with DMEM-

F12 supplemented with 10% FBS and 1% antibiotic/antimycotic. Astrocytes were used after two passages. For experiments, primary astrocytes were used at 14 DIV.

The interaction between neurons and astrocytes was studied with co-cultures of the two cell types. Freshly isolated neurons were plated on top of a semi-confluent (70%) layer of astrocytes (DIV 7-12) in 12-well plates. Astrocytes were pre-conditioned to Neurobasal media for 24 hrs prior to addition of neurons. After 4 DIV, half of the Neurobasal media was replaced with media lacking glutamic acid. Co-cultures were used in experiments 10 days following addition of neurons.

3.4 Nucleoside Release Assays

All experiments with primary neurons, astrocytes or co-cultures used physiological buffer in 12-well plates. Physiological buffers contained a final concentration of 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 2.9 mM KCl, 1.2 mM $MgCl_2$, 4.9 mM KCl, 1.4 mM KH_2PO_4, 1 mM $CaCl_2$, 118 mM NaCl, and 11 mM glucose, and were made to final pH of 7.4, using NaOH or HCl, and an osmolarity of 300 ± 10 mOsm. Cells were washed twice with physiological buffer and then incubated with 13.7 kBq [^{3}H]ADE for 30 min at 37^{o}C. The [^{3}H]ADE is taken up by cells and is metabolized to [^{3}H] adenine nucleotides (Sinclair et al., 2000). Cells were washed twice again, to remove extracellular tritium. In experiments with cultured neurons or co-cultures, cells were treated in 0.5 ml of control (buffer), NMDA (100 µM), the ENT1 and ENT2 inhibitor - DPR (30µM), the eN inhibitor AOPCP (50 µM) or the selective ENT1 inhibitor NBMPR (100nM) for 30 min at 37^{o}C. After incubation, the supernatants were

collected and 0.4 ml was quantified for extracellular radioactivity by scintillation spectrometry. Cells were dissolved in 0.35 ml 1M NaOH overnight at 37^{o}C, 0.2 ml of the cell lysate was counted for intracellular radioactivity and 0.15 ml for protein content.

It has been previously shown that under ATP depleting conditions, astrocytes release AN which is then metabolized to ADO (Parkinson and Xiong, 2004). To examine the importance of CD73 for the extracellular appearance of ADO, astrocyte cultures from C57Bl6 and CD73 KO mice were used. Astrocytes were washed with buffer twice before incubation with [^{3}H]ADE for 30 min at 37^{o}C. Astrocytes were then treated with buffer (control), glucose deprivation (GD) or oxygen glucose deprivation conditions (OGD). GD was obtained by treating cells for 30 min at 37^{o}C with buffer in which glucose was replaced with 2DG (10mM) to inhibit glycolysis and oxidative phosphorylation. For OGD treatment, cells were treated with 2DG containing buffer and were placed in a humidified chamber containing 95% N_2 and 5% CO_2 for 1 hour at 37^{o}C. Oxygen content was monitored with ProOx 110 controller and maintained at 2%. [^{3}H]Purine release from astrocytes was tested with eN inhibitor AOPCP (50µM) and ENT1 and ENT2 inhibitor DPR (30µM).

3.5 Quantification of Purines

To identify purines released from astrocytes and neurons, supernatants (20 µl) were combined with cold carrier (5 µl) containing AN, INO, HX and ADO, each at 15 mM and separated by thin-layer chromatography (TLC) as described by Schrader and Gerlach (1976). Samples were spotted on gel-silica plates. Separation was done in solvent

containing n-butanol, ethyl acetate, methanol and ammonium hydroxide (7: 4: 3: 4). Purines separated in the following order, from the bottom: AN → INO → HX → ADO. This method does not separate ATP, ADP or AMP they run together as AN. Spots were visualized under UV light, marked, scraped off the plates and dissolved in 500µl 0.2 M HCl for 1 hour. Scintillation fluid (5mL) was added to determine radioactivity by scintillation spectrometry.

3.6 Ecto 5'-nucleotidase Assay

eN activity was assessed from wild type (C57bl6) and knockout (CD73 KO) cell cultures and tissue samples. For cell cultures, primary astrocytes were grown on 12 well plates. The medium was aspirated from wells and cells were washed twice with buffer. Cells were then incubated with 30 µM DPR in buffer for 15 min at room temperature. Following this, 1.85 kBq [^{14}C]AMP (10 µM) containing 30 µM DPR with or without 50 µM AOPCP was added to cells for 10 min at room temperature. DPR was included in the assays to minimize cellular uptake of any [^{14}C]ADO formed. After incubation the extracellular medium was extracted and assayed for [^{14}C]purines by TLC and scintillation spectroscopy. Cells were lysed with 1.0M NaOH and measured for intracellular [^{14}C] purines and protein content.

To determine eN activity in brain tissue, cortices were homogenized in 0.32 M sucrose with glass/Teflon homogenizer. The homogenate was centrifuged at 1000g for 10 min and the pellet was washed twice in 0.32 M sucrose solution. The supernatants were collected at the end of each wash step, up to three times. The pooled supernatant

was centrifuged a final time at 20,000g for 45 min at 4°C. Following this, the supernatant was discarded and the pellet was resuspended in HEPES buffer (110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM CaCl, 1.0 mM $MgSO_4$ and 20mM HEPES, pH 7.4) and then assayed for protein content. Samples were stored at –80°C.

Tissue eN assay was performed with total reaction volume of 0.3 ml. This mixture consisted of 0.1ml cortex membrane protein, prepared to final concentrations of 10 µg/ml, 50 µg/ml and 100µg/ml, 0.1ml [^{14}C]AMP (300µM) and 0.1 ml of buffer with or without AOPCP (50 µM). After 10 min incubation, samples were centrifuged for 2 min to collect supernatant to assess radioactivity and [^{14}C] purine content by TLC.

3.7 Statistical Analysis

All data are reported as means ± SEM. Data were obtained from a minimum of three independent experiments, each completed in triplicate. Statistical significance was determined by one-way ANOVA. Post hoc analyses with Dunnett's or Tukey's post hoc tests were used to compare control to other experimental conditions. In some cases differences between two means was determined with student's t-tests. Two way ANOVAs were used to compare two factors (i.e. cell-type or co-culture) with multiple observations (i.e. purines). All graphs and statistics generated were done on GraphPad Prism version 5.01.

Chapter 4: Results

4.1 Activation of the NMDA receptor evoked ADO and INO release from Tg hENT1 neurons.

Transgenic (Tg) hENT1 cortical neurons were cultured to assess release of [^{3}H]purines AN, ADO, INO, and HX. Basal levels of extracellular purines from CD1 and hENT1 neurons are outlined in Table 4.1. The most abundant purine in neuron cultures was INO, followed by AN and HX. INO, HX and ADO were comparable between cell types, however AN was significantly greater in Wt than Tg cells.

In both CD1 and hENT1 neurons, NMDA-treatment stimulated INO and ADO release (Figure 4.1; Table 4.2). NMDA-evoked ADO release from Tg neurons was greater than CD1 neurons (0.74 vs. 0.51 pmol/mg; Table 4.2). INO levels were also significantly increased by NMDA (1.69 vs. 1.27 pmol/mg; $p < 0.001$; Table 4.2). The ENT1 and ENT2 inhibitor DPR attenuated these NMDA-induced increases. The eN inhibitor AOPCP did not decrease NMDA-evoked INO or ADO release in CD1 and hENT1 neurons. The selective inhibitor of ENT1, NBMPR also did not affect NMDA induced ADO or INO release (Figure 4.2). Between the two cell types, control levels of ADO or INO were comparable, although the extent of NMDA stimulation in hENT1 neurons was far greater in hENT1 than CD1 neurons (Table 4.3 and 4.4).

In wt neurons, there were no differences in the levels of AN and HX when stimulated with NMDA, also the effect of DPR, AOPCP or NBMPR did not decrease AN or HX levels (Figure 4.3). Conversely, with Tg hENT1 neurons, NMDA significantly increased levels of AN and HX. In some experiments, AN was also increased by NMDA. Between

wt CD1 and Tg hENT1 neurons there were no significant differences in the levels of AN or HX when stimulated with NMDA (Table 4.3 and 4.4). Figure 4.3 shows some differences between CD1 and Tg hENT1.

Table 4.1: Basal levels of AN, INO, HX and ADO, released from CD1 and hENT1 neurons

[^{3}H]Purines	CD1	hENT1	P-value
AN	0.30 ± 0.03	0.16 ± 0.01	**P<0.01
ADO	0.14 ± 0.02	0.11 ±0.01	P > 0.05
INO	0.39 ± 0.03	0.33 ±0.03	P > 0.05
HX	0.13 ± 0.01	0.10 ±0.01	P > 0.05

Neurons were pre-incubated with [^{3}H]ADE. Extracellular media was collected from primary cultured neurons in the control condition after 30 min at 37^{o}C. Samples were analyzed for AN, ADO, INO and HX. Data are expressed as means (pmol/mg) ± SEM (n = 63). Data were analyzed by two-way ANOVA with Bonferroni post hoc analysis; ** p < 0.01.

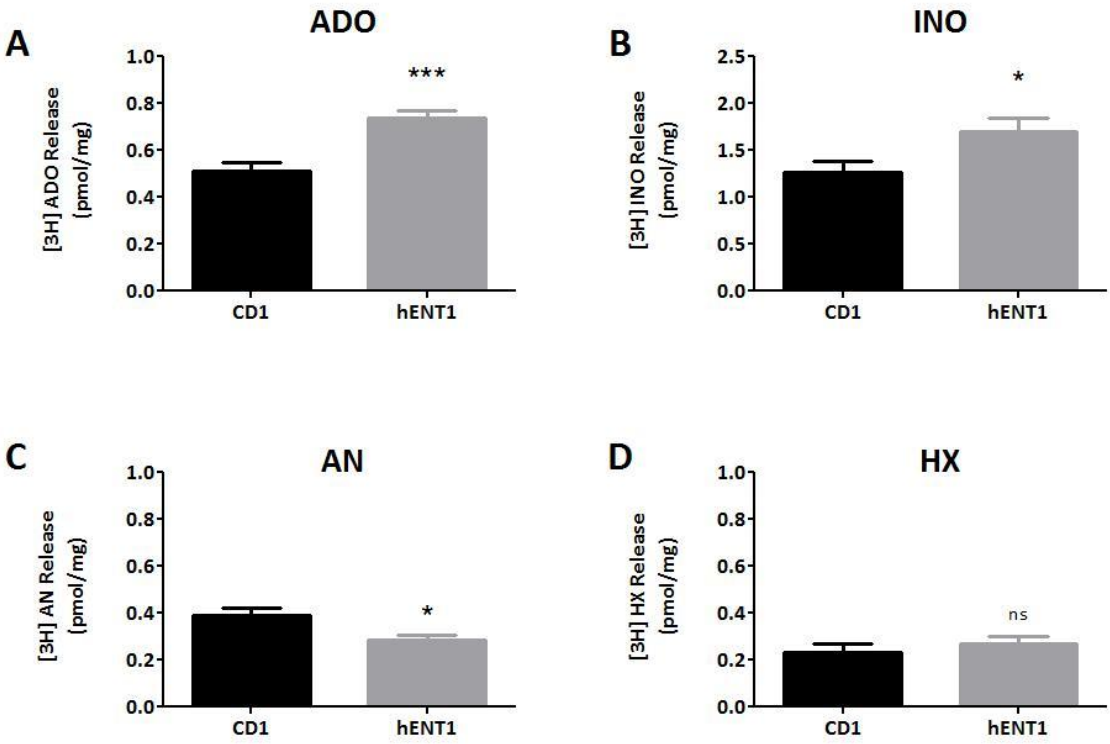

A
ADO
[3H] ADO Release (pmol/mg)
1.0
0.8
0.6
0.4
0.2
0.0
***
CD1
hENT1
B
INO
[3H] INO Release (pmol/mg)
2.5
2.0
1.5
1.0
0.5
0.0
*
CD1
hENT1
C
AN
[3H] AN Release (pmol/mg)
1.0
0.8
0.6
0.4
0.2
0.0
*
CD1
hENT1
D
HX
[3H] HX Release (pmol/mg)
1.0
0.8
0.6
0.4
0.2
0.0
ns
CD1
hENT1

Figure 4.1: The effect of NMDA-evoked A) ADO, B) INO, C) AN and D) HX release from wt CD1 and Tg hENT1 neurons.

Cells were pre-incubated with [^{3}H]ADE for 30 min at 37^{o}C. Neurons were stimulated with 100µM NMDA for another 30 min. The supernatants were collected and assayed for [^{3}H]purines by TLC and scintillation spectroscopy. Data are expressed as means (pmol/mg) ± SEM (n = 62-63). Differences between the two means were determined by Student's T test; *** $p < 0.001$, * $p < 0.05$.

Table 4.2: The average NMDA-evoked [^{3}H]purine release from CD1 and hENT1 neurons

[^{3}H]purines	CD1	hENT1	P-value
AN	0.39 ± 0.03	0.28 ± 0.03	* P < 0.05
ADO	0.51 ± 0.03	0.74 ± 0.01	*** P < 0.001
INO	1.27 ± 0.12	1.69 ± 0.02	* P < 0.05
HX	0.23 ± 0.04	0.27 ± 0.08	P > 0.05

Neurons were pretreated with [^{3}H]ADE for 30 min and then treated with 100µM NMDA at 37^{o}C. Samples were taken after 30 min, separated by TLC and analyzed by scintillation spectroscopy. Data are expressed as means (pmol/mg) ± SEM (n = 62-63). Data were analyzed by Student's T test; *** $p < 0.001$, * $p < 0.05$.

Table 4.3: Extracellular values ([^{3}H]purines) from CD1 neuron release experiments.

Treatment	AN(pmol/mg)	ADO(pmol/mg)	INO(pmol/mg)	HX(pmol/mg)
Control	0.27±0.05	0.17±0.05	0.49±0.08	0.16±0.02
DPR	0.32±0.08	0.07±0.02	0.15±0.04	0.15±0.02
NMDA	0.44 ±0.07	0.58 ± 0.07***	1.48±0.18***	0.21±0.03
DPR + NMDA	0.45 ±0.07	0.13±0.02+++	0.30±0.05+++	0.24±0.03
Control	0.28±0.07	0.12±0.02	0.32±0.04	0.12±0.02
AOPCP	0.28±0.07	0.11±0.01	0.31±0.04	0.12±0.02
NMDA	0.33±0.06	0.50±0.05***	1.06±0.17***	0.19±0.03
AOPCP + NMDA	0.36±0.05	0.43±0.06***	0.93±0.15**	0.17±0.03
Control	0.36±0.06	0.13±0.01	0.36±0.03	0.11±0.02
NBMPR	0.33±0.05	0.10±0.01	0.35±0.03	0.11±0.02
NMDA	0.39±0.05	0.46±0.05***	1.27±0.21 ***	0.30±0.10
NBMPR + NMDA	0.38±0.05	0.52±0.03***	1.40±0.21***	0.22±0.03

Primary cultured neurons were pre-treated with [^{3}H]ADE for 30 min. At 37^{o}C, CD1 neurons were treated with 30 µM DPR, 50 µM AOPCP or 100nM NBMPR alone or in combination with 100 µM NMDA. The extracellular media was collected and analyzed for [^{3}H]purines. Data are expressed as means (pmol/mg) ± SEM (n = 20-21). Analysis was performed by one-way ANOVA and post-hoc analysis with Tukey's tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine and +++ $p < 0.001$, ++ $p < 0.01$ or + $p < 0.05$ relative to NMDA treatment.

Table 4.4: Extracellular values ([^{3}H]purines) from hENT1 neuron release experiments.

Treatment	AN(pmol/mg)	ADO(pmol/mg)	INO(pmol/mg)	HX(pmol/mg)
Control	0.18±0.03	0.11±0.01	0.36±0.05	0.12±0.02
DPR	0.14±0.01	0.03±0.01	0.07±0.01	0.13±0.01
NMDA	0.29±0.04	0.72±0.05***	1.67±0.28***	0.43±0.07***
DPR + NMDA	0.33±0.05*	0.17±0.02+++	0.34±0.06+++	0.23±0.02++
Control	0.13±0.02	0.10±0.01	0.29±0.04	0.09±0.01
AOPCP	0.11±0.02	0.09±0.01	0.27±0.04	0.09±0.01
NMDA	0.23±0.04	0.74±0.07***	1.67±0.28***	0.18±0.03**
AOPCP + NMDA	0.30±0.04***	0.68±0.07***	1.69±0.23***	0.19±0.02**
Control	0.17±0.01	0.11±0.01	0.34±0.04	0.09±0.01
NBMPR	0.14±0.01	0.10±0.01	0.31±0.04	0.09±0.01
NMDA	0.33±0.04**	0.75±0.04***	1.74±0.26***	0.20±0.02**
NBMPR + NMDA	0.31±0.04**	0.70±0.04***	1.84±0.27***	0.22±0.02***

Primary cultured neurons were pre-treated with [^{3}H] ADE for 30 min. hENT1 neurons were treated with 30 µM DPR, 50 µM AOPCP or 100nM NBMPR alone or in combination with 100 µM NMDA. The extracellular media was collected and analyzed for [^{3}H]purines. Data are expressed as means (pmol/mg) ± SEM (n = 21). Analysis was performed by one-way ANOVA and post-hoc analysis with Tukey's tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine and +++ $p < 0.001$, ++ $p < 0.01$ or + $p < 0.05$ relative to NMDA treatment.

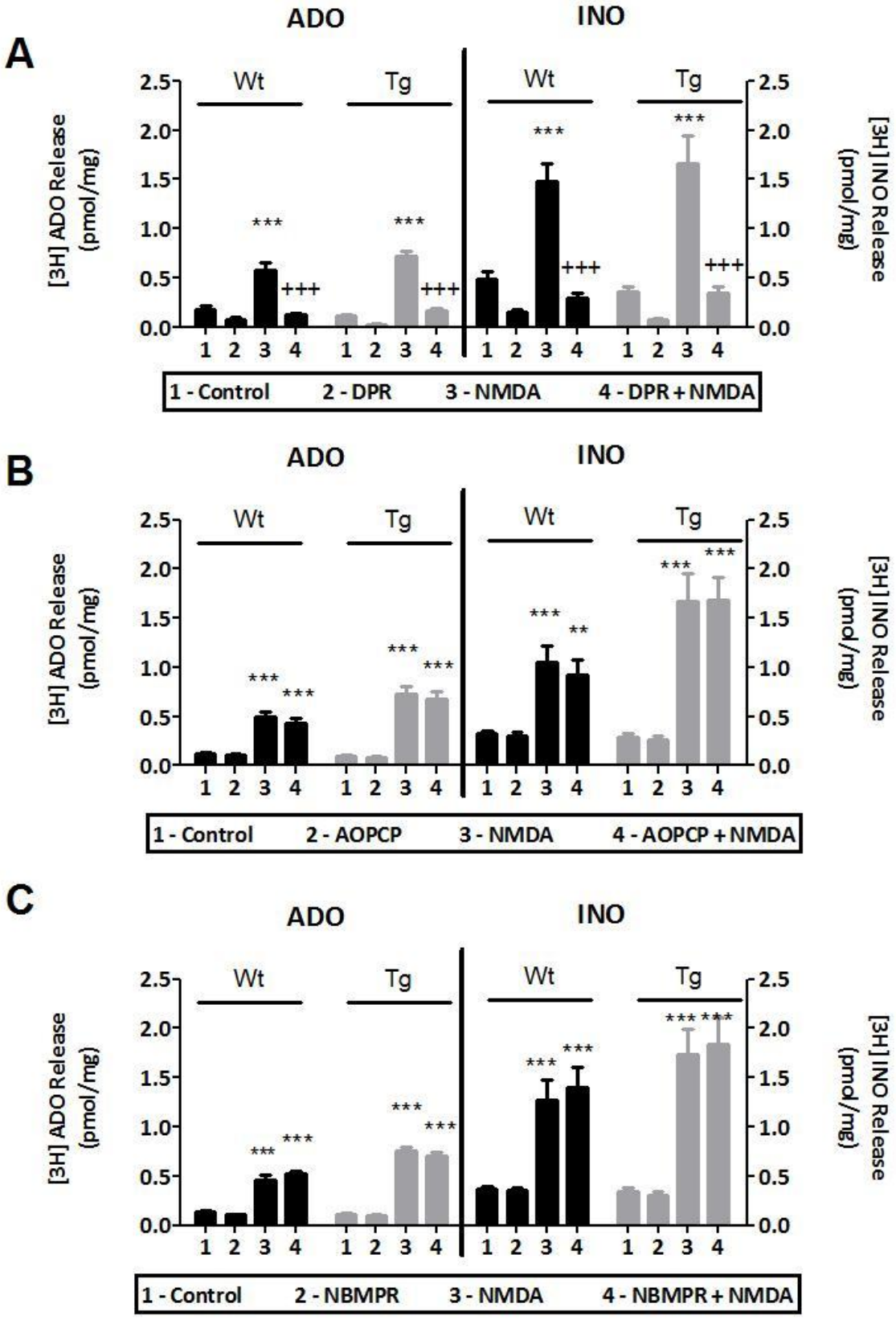

A
ADO
INO
Wt
Tg
Wt
Tg
[3H] ADO Release (pmol/mg)
[3H] INO Release (pmol/mg)
2.5
2.0
1.5
1.0
0.5
0.0
***
+++
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
1 - Control 2 - DPR 3 - NMDA 4 - DPR + NMDA
B
ADO
INO
Wt
Tg
Wt
Tg
[3H] ADO Release (pmol/mg)
[3H] INO Release (pmol/mg)
***
**
1 - Control 2 - AOPCP 3 - NMDA 4 - AOPCP + NMDA
C
ADO
INO
Wt
Tg
Wt
Tg
[3H] ADO Release (pmol/mg)
[3H] INO Release (pmol/mg)
***
1 - Control 2 - NBMPR 3 - NMDA 4 - NBMPR + NMDA

Figure 4.2: Comparison between ADO and INO release from wt CD1 and Tg hENT1 neurons.

Cells were pre-incubated with [^{3}H]ADE and then with treatment A) DPR, B) AOPCP or C) NBMPR with or without 100 µM NMDA. Extracellular fluid was collected and analyzed by TLC and scintillation spectroscopy. Data are expressed as mean ± SEM (n = 20-21). Statistical analysis between control and treatment groups for wt CD1 or Tg hENT1 neurons was performed by one-way ANOVA and post hoc analysis with Tukey's tests. *** $P < 0.001$; ** $P < 0.05$, * $P < 0.01$ compared with CD1 or hENT1 treatment control. +++ $P < 0.001$ compared to CD1 NMDA or hENT1 NMDA treatment.

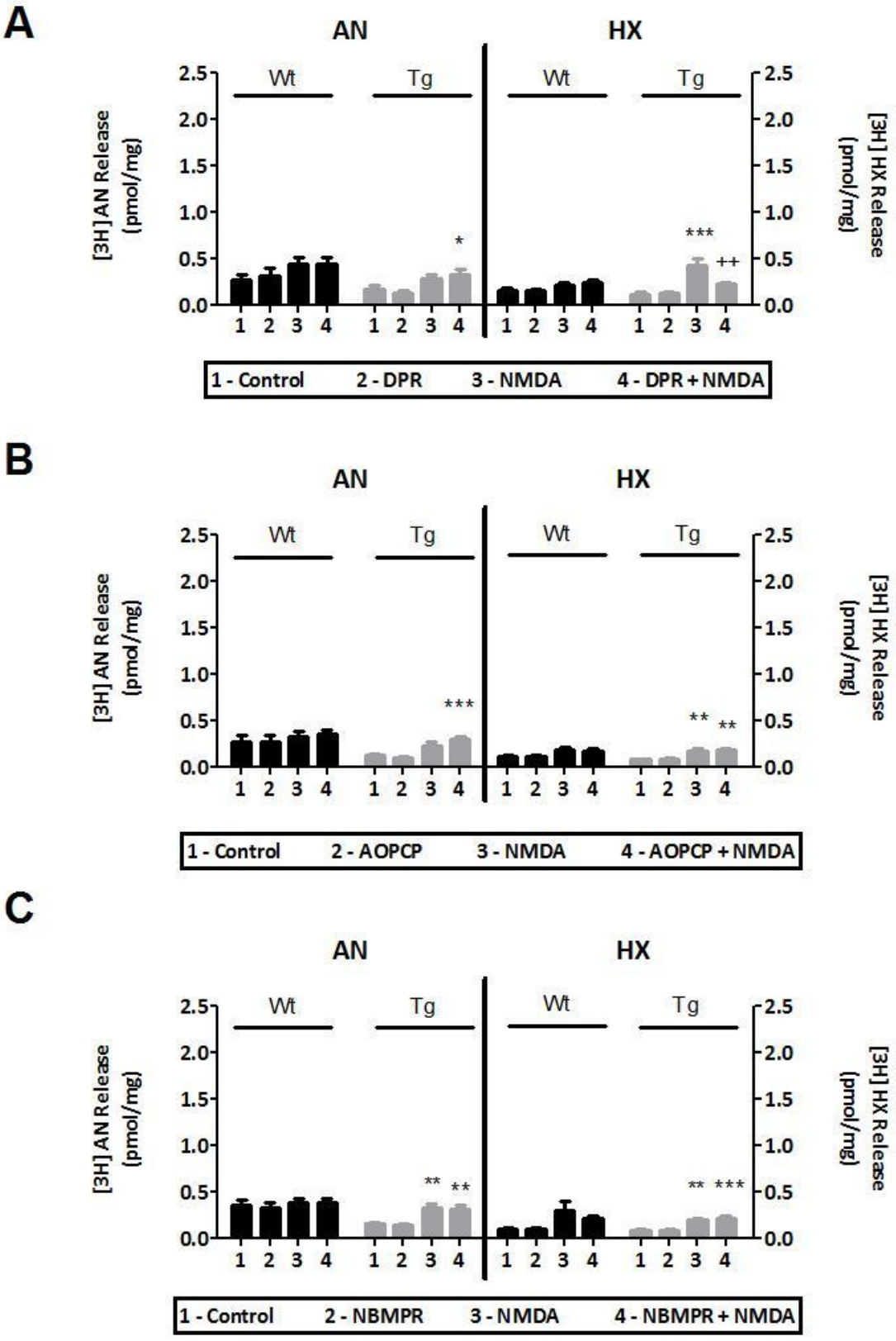

A
AN
HX
Wt
Tg
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
*
***
++
1 - Control 2 - DPR 3 - NMDA 4 - DPR + NMDA
B
AN
HX
Wt
Tg
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
***
** **
1 - Control 2 - AOPCP 3 - NMDA 4 - AOPCP + NMDA
C
AN
HX
Wt
Tg
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
** **
** ***
1 - Control 2 - NBMPR 3 - NMDA 4 - NBMPR + NMDA

Figure 4.3: Comparison between AN and HX release from wt CD1 and Tg hENT1 neurons.

Cells were pre-incubated with [^{3}H]ADE and then with treatment A) DPR, B) AOPCP or C) NBMPR with or without 100 µM NMDA. Extracellular fluid was collected and analyzed by TLC and scintillation spectroscopy. Data are expressed as mean ± SEM (n = 20-21). Statistical analysis between control and treatment groups for wt CD1 or Tg hENT1 neurons was performed by one-way ANOVA and post hoc analysis with Tukey's tests. *** $P < 0.001$; ** $P < 0.05$, * $P < 0.01$ compared with CD1 or hENT1 treatment control. ++ $P < 0.01$ compared to CD1 NMDA or hENT1 NMDA treatment.

4.2 AOPCP inhibits ADO production in wt C57Bl6 but not in knockout CD73 astrocytes.

We examined the activity of membrane bound eN in brain tissue and cell culture of wt C57bl6 (C57) and knockout CD73 (CD73 KO) astrocytes. The eN catalyzes the last step from AMP to ADO in the extracellular pathway. In figure 4.4, wt astrocyte cultures, eN was able to convert [^{14}C]AMP to [^{14}C]ADO, although this action was inhibited by AOPCP. CD73 KO astrocytes lack expression of eN. As expected the [^{14}C]ADO levels from control were low (0.13 ± 0.01 pmol/mg) and did not significantly change in the presence of AOPCP (0.10 ± 0.01 pmol/mg).

In tissue eN assays, membrane proteins were extracted from cortical tissues from wt C57 and CD73 KO mice. Figure 4.5 shows the levels of [^{14}C]purines AN, INO, HX and ADO in both wt C57 and CD73 KO tissues. The effect of AOPCP to decrease ADO levels was seen in wt C57 protein samples. However in CD73 KO samples, ADO formation was low and AOPCP had no effect to decrease it. Three different protein concentrations were assayed for ecto-5'nucleotidase activity with similar results.

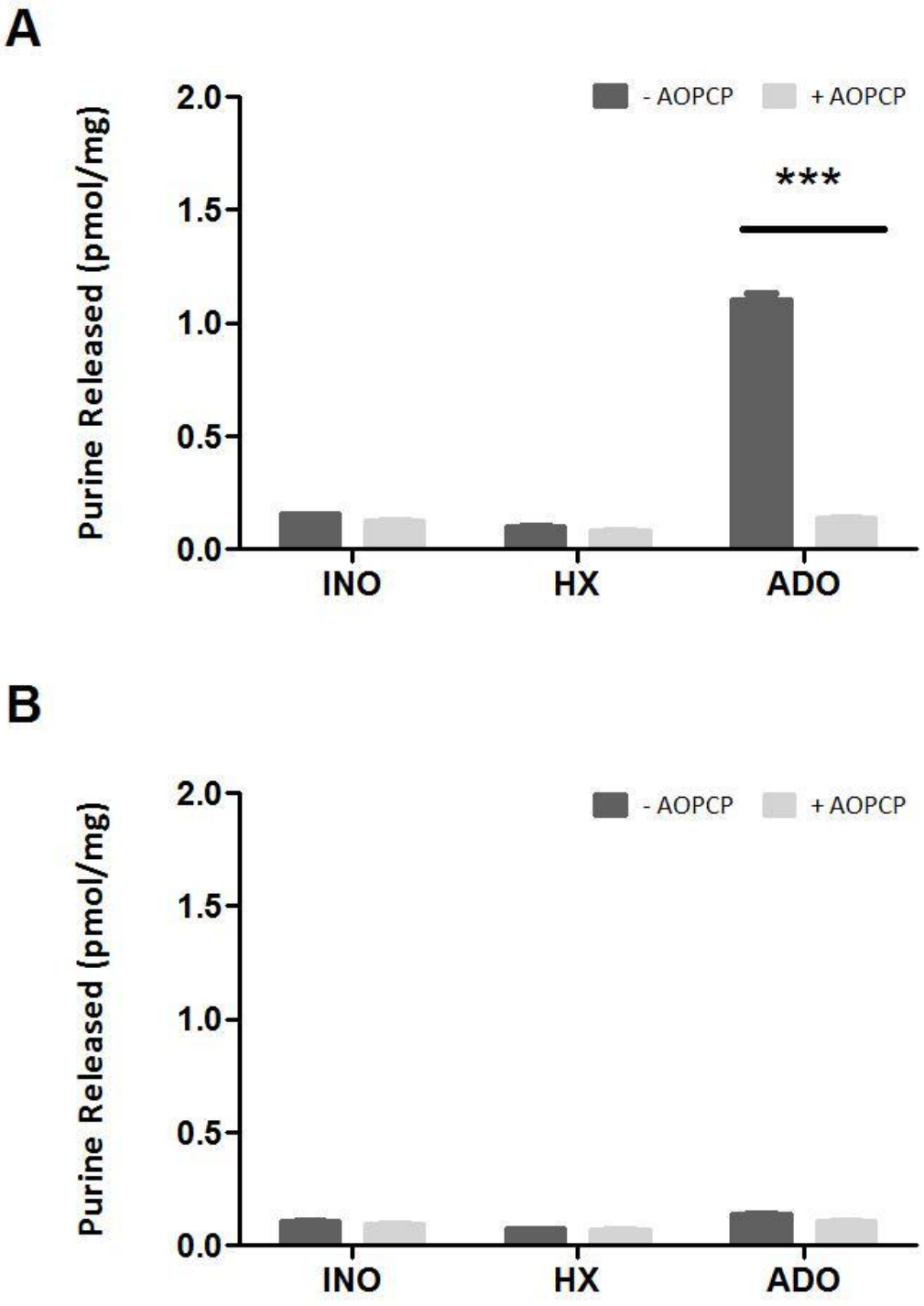

A
- AOPCP
+ AOPCP
***
Purine Released (pmol/mg)
2.0
1.5
1.0
0.5
0.0
INO
HX
ADO
B
- AOPCP
+ AOPCP
Purine Released (pmol/mg)
2.0
1.5
1.0
0.5
0.0
INO
HX
ADO

Figure 4.4: Ecto-5’ nucleotidase assay from A) wt C57 and B) CD73 knockout primary cultured astrocytes.

Cells were first treated with 30 µM DPR for 15 min. Astrocytes were then treated for 10 minutes with a reaction mixture containing final concentrations of 10 µM [^{14}C]AMP, 30 µM DPR in buffer with or without 50 µM AOPCP. Afterwards, supernatant was collected for TLC and quantified by scintillation spectrometry. Data are expressed as mean ± SEM (n = 18). Statistical analysis between treatment groups was performed by student’s T test. *** $P < 0.001$.

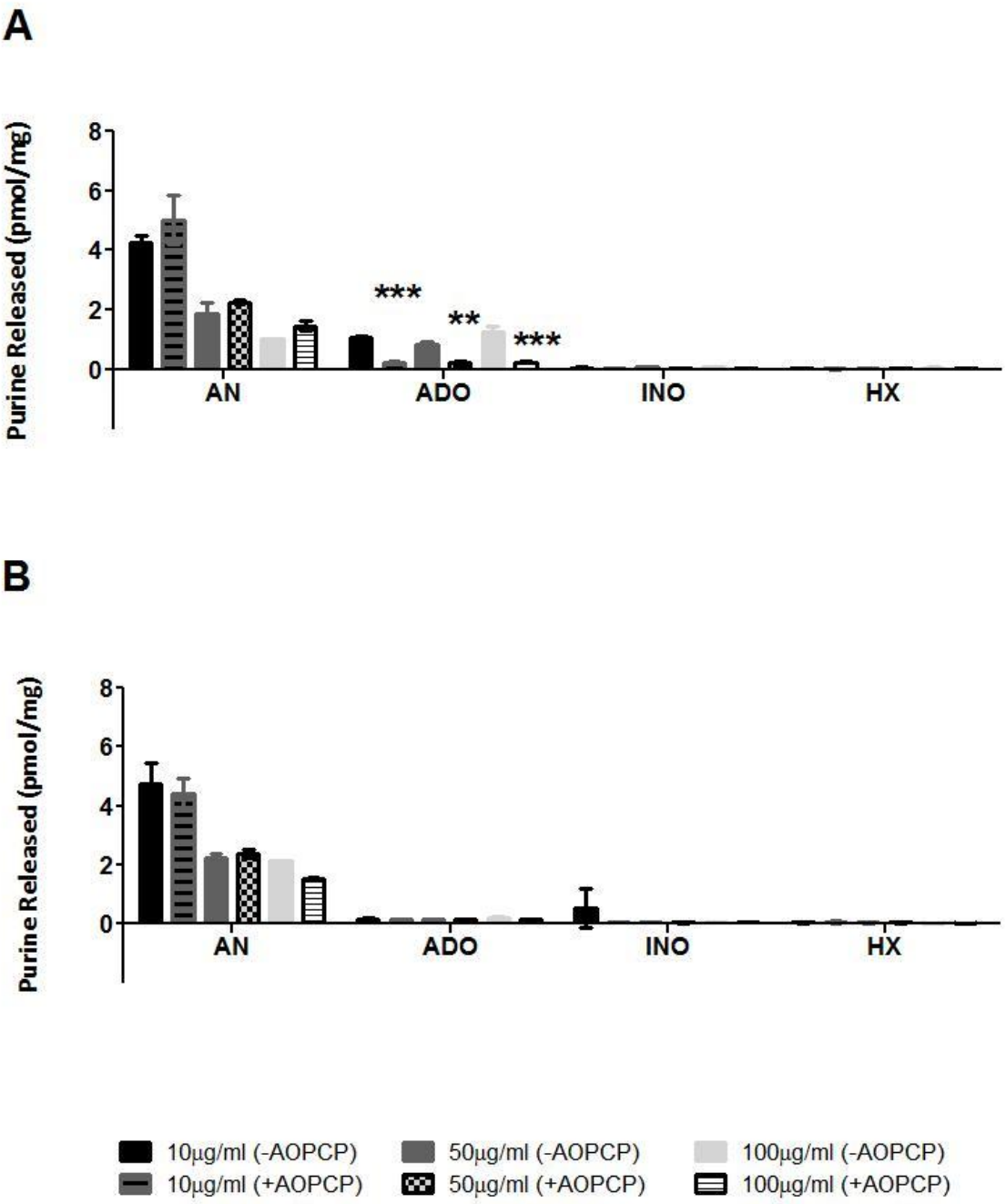

A
Purine Released (pmol/mg)
8
6
4
2
0
***
**
***
AN
ADO
INO
HX
B
Purine Released (pmol/mg)
8
6
4
2
0
AN
ADO
INO
HX
10μg/ml (-AOPCP)
10μg/ml (+AOPCP)
50μg/ml (-AOPCP)
50μg/ml (+AOPCP)
100μg/ml (-AOPCP)
100μg/ml (+AOPCP)

Figure 4.5: Ecto-5' nucleotidase assay from cortical tissues from A) wt C57 and B) CD73 KO mice.

Membrane proteins were extracted from tissue and 10 µg/ml, 50µg/ml, or 100µg/ml of protein was assayed. After 10 min incubation, with or without AOPCP and [^{14}C]AMP, the extracellular supernatant was collected for AN, INO, HX and ADO by TLC and quantified by scintillation spectroscopy. Analysis was performed by one-way ANOVA with Tukey's post hoc analysis. Data are expressed as means ± SD (n = 2). *** $P < 0.001$, ** $p < 0.01$

4.2.1 CD73 Knockout astrocytes produce less ADO, more AN and similar INO and HX levels in comparison to wt astrocytes.

Primary cultured astrocytes from wt C57 and CD73 KO mice were compared to assess AN, INO, HX and ADO release under physiological buffer (control), GD or OGD conditions. As shown previously by Parkinson et al 2004 using Sprague Dawley rat, astrocytes produce ADO by releasing AN with subsequent dephosphorylation reactions by eN. Figure 4.6 compares the extracellular levels of total purines from wt C57 and CD73 KO astrocytes when treated with control, GD or OGD conditions. Relative to buffer treatment, [^{3}H]purines released by both types of astrocytes increased with metabolic stress. There was no significant difference in total purine release between wt and CD73 KO astrocytes.

Table 4.5, further breaks down the amount of [^{3}H]purines AN, INO, HX or ADO in control, GD or OGD conditions. Compared to wt C57 astrocytes, the CD73 KO cells produced significantly less ADO in all three conditions. There were no significant cell type differences in INO or HX release. A trend towards higher levels of AN in KO relative to wt astrocytes was observed in control and GD conditions; this difference was statistically significant in OGD conditions.

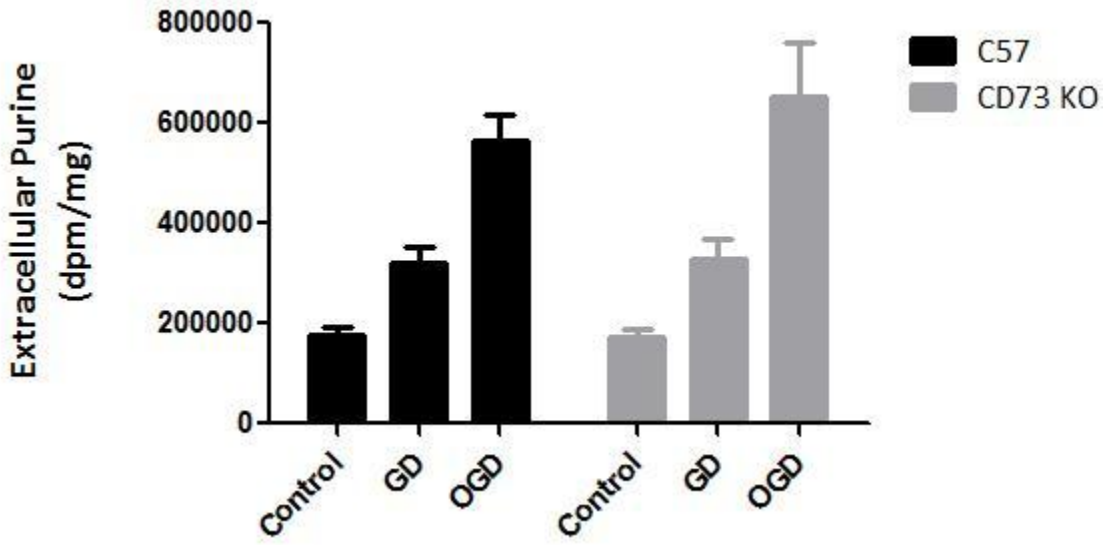

Extracellular Purine
(dpm/mg)
800000
600000
400000
200000
0
Control
GD
OGD
Control
GD
OGD
C57
CD73 KO

Figure 4.6: Total extracellular [^{3}H]purines (dpm/mg) in control, GD, or OGD conditions.

Extracellular media was collected after 30 min (control, GD) or 1 hour (OGD) from wt C57 or CD73 KO astrocytes. Total [^{3}H]purines released was counted using a scintillation spectrometer. Data are expressed as mean ± SEM (n = 20). Statistical analysis between control and treatment groups for wt C57 or CD73 KO astrocytes was performed by two-way ANOVA and Bonferroni's post hoc analysis. No significant differences were detected between wt C57 and CD73 KO astrocytes.

Table 4.5: Extracellular [^{3}H]purine levels of AN, INO, HX and ADO, released from wt C57 or CD73 KO astrocytes under control, GD or OGD conditions.

	Control		GD		OGD	
	C57	CD73 KO	C57	CD73 KO	C57	CD73 KO
AN	1.17±0.13	1.25±0.14	1.07±0.12	1.29±0.13	0.98±0.11	1.80±0.32 **
ADO	0.38±0.02	0.06±0.07 ***	0.39±0.03	0.06±0.01 ***	0.70±0.06	0.09±0.01 ***
INO	0.36±0.02	0.40±0.05	1.52±0.19	1.49±0.28	3.96±0.53	3.63±0.74
HX	1.04±0.09	1.21±0.12	2.52±0.30	2.08±0.24	5.62±0.72	4.40±0.64

Extracellular media was collected from wt C57 or CD73 KO astrocytes in control (buffer), GD or OGD conditions. [^{3}H]Purines was separated by TLC and analyzed by scintillation spectrometer. Data are expressed as mean ± SEM (n = 20). Statistical analysis between control and treatment groups for wt C57 or CD73 KO astrocytes was performed by two-way ANOVA and Bonferroni's post hoc analysis. *** $p < 0.001$, ** $p < 0.01$, relative to wt.

In figure 4.7, control levels of ADO in wt C57 were significantly higher than CD73 KO (0.38 ± 0.02 vs. 0.06 ± 0.01 pmol/mg; Table 4.5). In wt C57 astrocytes, inhibition of ENT1 transporter did not decrease ADO levels; rather it elevated ADO to 0.54 ± 0.05 pmol/mg. In CD73 KO astrocytes, DPR did not significantly change ADO levels (0.05 ± 0.01 pmol/mg). Inhibition of eN with AOPCP did significantly decrease ADO levels in wt C57 astrocytes to 0.18 ± 0.02 pmol/mg. However, in CD73 KO astrocytes, ADO levels were not significantly altered.

In GD conditions (Figure 4.7b), ADO levels did not change, relative to control conditions in both wt C57and CD73 KO astrocytes. In wt C57 astrocytes, the effect of DPR elevated ADO levels but this was not statistically significant. AOPCP significantly decreased ADO levels. In CD73 KO astrocytes, ADO levels were not affected by DPR or AOPCP treatment.

Under OGD conditions (Figure 4.7c), ADO levels, in C57 astrocytes were elevated to 0.70 ± 0.06 pmol/mg. With the addition of DPR, ADO levels significantly increased to 1.00 ± 0.08 pmol/mg. Conversely, the presence of AOPCP decreased ADO levels to 0.24 ± 0.02 pmol/mg. In contrast, ADO levels in CD73 KO astrocytes were unaffected by OGD conditions and also unaltered by the presence of DPR or AOPCP.

In astrocytes, the main pathway of ADO formation is through the extracellular pathway; AN are released into the extracellular environment and then dephosphorylated to ADO. Wt C57 astrocytes showed a decreasing trend in AN levels in control, GD, and OGD conditions (Table 4.5 and 4.6). For CD73 KO astrocytes, AN levels

were unaffected by GD but were significantly increased by OGD (Table 4.5 and 4.7). The ENT1 inhibitor, DPR did not affect AN levels in either cell type. In the presence of AOPCP, AN levels were significantly increased in wt C57 astrocytes in control, GD and OGD conditions. However in CD73 KO astrocytes, AOPCP did not affect AN levels (Figure 4.7).

INO levels were similar between wt C57 and CD73 KO cells, in all three treatment conditions. However, INO was significantly increased by GD and OGD in both cell types (Table 4.5, 4.6 and 4.7). In both cell types, levels of INO were significantly decreased in the presence of DPR but not affected by AOPCP (Figure 4.8; Tables 4.6 and 4.7). Similarly, levels of HX were similar between wt and KO cells in all three treatment conditions (Figure 4.8; Table 4.6 and 4.7). In wt, DPR produced a significant decrease in HX in control, but not in GD or OGD conditions; this was not observed in CD73 KO astrocytes. In wt cells, AOPCP had no effect on HX levels, but in CD73 KO cells, AOPCP produced a significant increase in HX in GD but not in control or OGD conditions.

Table 4.6: Extracellular [^{3}H]purines from wt C57 astrocyte release from basal, GD or OGD conditions.

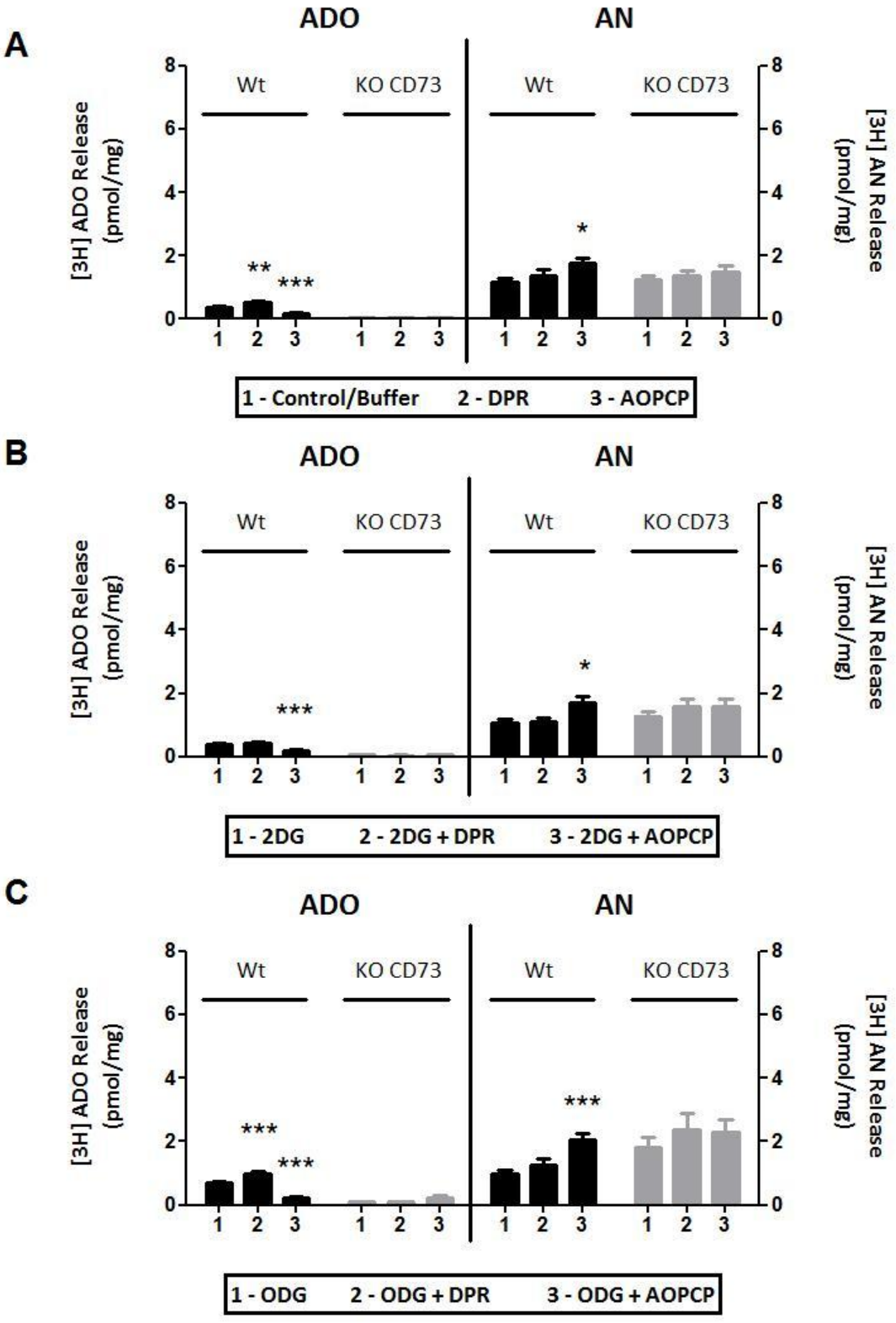
Treatment	AN(pmol/mg)	ADO(pmol/mg)	INO(pmol/mg)	HX(pmol/mg)
Control	1.17±0.13	0.38±0.02	0.36±0.02	1.04±0.09
DPR	1.39±0.18	**0.54±0.05	***0.14±0.01	*0.71±0.09
AOPCP	*1.76±0.19	***0.18±0.02	0.36±0.03	1.14±0.10
GD	1.07±0.12	0.39±0.03	1.52±0.19	2.52±0.30
GD + DPR	1.09±0.15	0.44±0.04	***0.18±0.03	1.82±0.13
GD + AOPCP	*1.69±0.21	***0.20±0.02	1.48±0.15	2.73±0.24
OGD	0.98±0.11	0.70±0.06	3.96±0.53	5.62±0.72
OGD + DPR	1.28±0.16	***1.00±0.08	***0.51±0.12	4.70±0.50
OGD + AOPCP	***2.067±0.18	***0.24±0.02	3.85±0.48	6.21±0.65

Extracellular media was collected from primary cultured wt C57 astrocytes in control (buffer), GD or OGD conditions. [^{3}H]Purines was separated by TLC and analyzed by scintillation spectrometer. Data are expressed as pmol/mg ± SEM (n=20). Analysis was performed by one-way ANOVA and post-hoc analysis with Dunnett's Multiple Comparison Test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine.

Table 4.7: Extracellular [^{3}H]purines from CD73 KO astrocyte release from basal, GD or OGD conditions.

Treatment	AN(pmol/mg)	ADO(pmol/mg)	INO(pmol/mg)	HX(pmol/mg)
Control	1.25±0.14	0.06±0.01	0.40±0.05	1.21±0.12
DPR	1.39±0.13	0.05z±0.01	0.08±0.01***	1.47±0.22
AOPCP	1.50±0.20	0.04±0.01	0.43±0.08	1.10±0.10
GD	1.29±0.13	0.06±0.01	1.49±0.28	2.08±0.24
GD + DPR	1.61±0.21	0.05±0.01	0.15±0.03**	1.95±0.25
GD + AOPCP	1.57±0.27	0.07±0.02	2.18±0.42	3.07±0.41*
OGD	1.80±0.32	0.09±0.01	3.63±0.74	4.40±0.64
OGD + DPR	2.36±0.54	0.11±0.02	0.32±0.06**	3.57±0.60
OGD + AOPCP	2.30±0.41	0.21±0.08	4.64±0.91	5.57±0.74

Extracellular media was collected from primary cultured CD73 KO astrocytes in control (buffer), GD or OGD conditions. [^{3}H]Purines was separated by TLC and analyzed by scintillation spectrometer. Data are expressed as pmol/mg ± SEM (n=18 - 20). Analysis was performed by one-way ANOVA and post-hoc analysis with Dunnett's Multiple Comparison Test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine.


A
ADO
AN
Wt
KO CD73
Wt
KO CD73
[3H] ADO Release (pmol/mg)
[3H] AN Release (pmol/mg)
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*
1 - Control/Buffer 2 - DPR 3 - AOPCP
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Wt
KO CD73
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KO CD73
[3H] ADO Release (pmol/mg)
[3H] AN Release (pmol/mg)
***
*
1 - 2DG 2 - 2DG + DPR 3 - 2DG + AOPCP
C
ADO
AN
Wt
KO CD73
Wt
KO CD73
[3H] ADO Release (pmol/mg)
[3H] AN Release (pmol/mg)
***
***
***
1 - ODG 2 - ODG + DPR 3 - ODG + AOPCP

Figure 4.7: Comparison between ADO and AN release from wt C57 or CD73 KO astrocyte cultures.

Cells were pre-incubated with [^{3}H] ADE and then treated with DPR or AOPCP in A) buffer (control) B) GD or C) OGD conditions. For control and GD conditions, extracellular fluid was collected after 30 min. In OGD conditions, the supernatants were collected after 1 hour in a humidified chamber containing 95% N_2 and 5% CO_2 at 37^{o}C. [^{3}H]Purines were separated by TLC and analyzed by scintillation spectroscopy. Analysis was done by one-way ANOVA with post hoc analysis using Dunnett's Multiple Comparison Test. Data are expressed as mean ± SEM (n = 18 - 20). *** $P < 0.001$; ** $P < 0.01$, * $P < 0.05$ compared with C57 or CD73 KO treatment control.

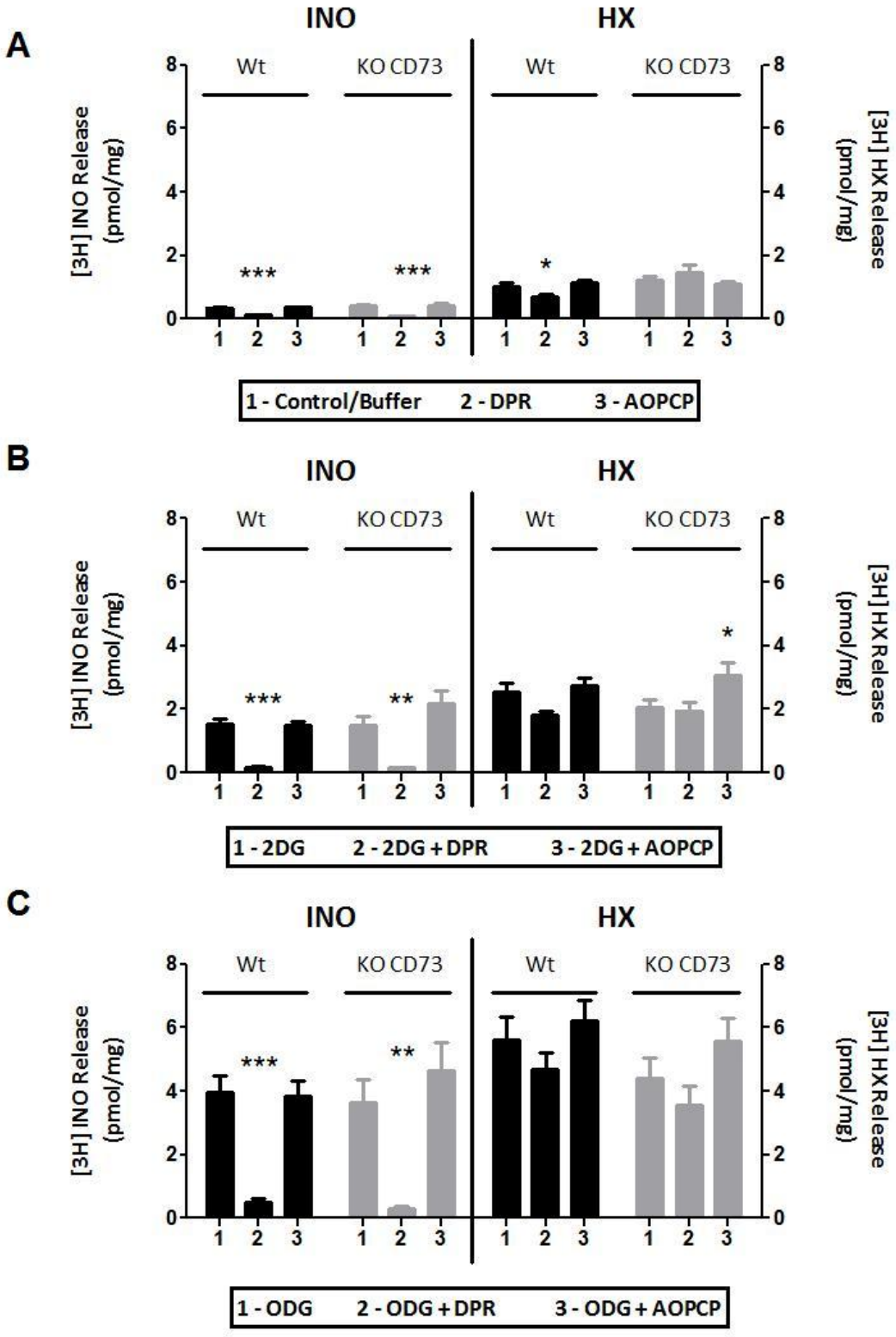

A
INO
HX
Wt
KO CD73
Wt
KO CD73
[3H] INO Release (pmol/mg)
[3H] HX Release (pmol/mg)
***
***
*
1 - Control/Buffer
2 - DPR
3 - AOPCP
B
INO
HX
Wt
KO CD73
Wt
KO CD73
[3H] INO Release (pmol/mg)
[3H] HX Release (pmol/mg)
***
**
*
1 - 2DG
2 - 2DG + DPR
3 - 2DG + AOPCP
C
INO
HX
Wt
KO CD73
Wt
KO CD73
[3H] INO Release (pmol/mg)
[3H] HX Release (pmol/mg)
***
**
1 - ODG
2 - ODG + DPR
3 - ODG + AOPCP

Figure 4.8: Comparison between INO and HX release from wt C57 or CD73 KO astrocyte cultures.

Cells were pre-incubated with [^{3}H] ADE and then treated with DPR or AOPCP in A) buffer B) GD or C) OGD conditions. For control and GD conditions, extracellular fluid was collected after 30 min; OGD was collected after 1 hour in humidified chamber containing 95% N_2 and 5% CO_2 at 37^{o}C. [^{3}H]Purines were separated by TLC and analyzed by scintillation spectroscopy. Analysis was done by one-way ANOVA with post hoc analysis using Dunnett's Multiple Comparison Test. Data are expressed as mean ± SEM (n = 18 - 20). *** $P < 0.001$; ** $P < 0.05$, * $P < 0.01$ compared with wt C57 or CD73 KO treatment control.

4.3 Role of ecto-5'nucleotidase in NMDA-evoked purine release from neuron-astrocyte co-cultures comprised of wild type (CD1) neurons and wt or CD73 KO astrocytes

To look at the role of eN in ADO formation, we established co-cultures using wt neurons (CD1) with wt (C57) or knockout astrocytes (CD73). Previous studies have shown that astrocytes affect the purine profile of release from neurons (Zamzow et al., 2008). Two co-cultures were compared 1) wild type (CD1-C57) and 2) eN deficient (CD1-CD73 KO).

Basal levels of [^{3}H]purines AN, INO, HX and ADO from wt and eN- co-cultures are outlined in Table 4.8. In eN deficient cultures, HX levels were significantly greater than in wt co-cultures ($p<0.001$). NMDA stimulation in rat co-cultures increases both INO and ADO levels (Zamzow et al., 2008a, b). From figure 4.9, the average levels of ADO in NMDA-treated wt co-cultures was 0.52 ± 0.05 pmol/mg and in eN deficient cultures was 0.38 ± 0.01 pmol/mg (Table 4.9). Statistical analysis showed that this difference was significant $P < 0.01$ (Figure 4.9). The average control levels for INO in wt (CD1-C57) and eN deficient (CD1-CD73 KO) was 0.77 ± 0.09 vs. 1.52 ± 0.12 pmol/mg respectively (Table 4.8). However NMDA-induced INO production showed no significant differences in INO release between wt (CD1-C57) and eN deficient (CD1-CD73 KO) cultures (Figure 4.9b).

Table 4.8: Average basal levels of [^{3}H]purines from wt (CD1-C57) and eN deficient (CD1-CD73) co-cultures.

[^{3}H]purines	CD1-C57	CD1 CD73 KO	P-value
AN	0.40±0.04	0.76±0.05	P > 0.05
ADO	0.16±0.02	0.16±0.01	P > 0.05
INO	0.77±0.09	1.52±0.12	P > 0.05
HX	2.19±0.36	5.82±0.60	***P < 0.001

Co-culture of neurons and astrocytes were pre-incubated with [^{3}H]ADE. Extracellular media was collected from both co-cultures in the control condition after 30 min at 37^{o}C. Samples were analyzed for AN, ADO, INO and HX. Data are expressed as means (pmol/mg) ± SEM (n = 27-33). Data were analyzed by two-way ANOVA with Bonferroni post hoc analysis; ** p < 0.01.

Table 4.9: Average extracellular NMDA-evoked [^{3}H]purine release from wt (CD1-C57) and eN deficient (CD1-CD73) co-cultures

[^{3}H]purines	CD1-C57	CD1-CD73 KO	P-value
AN	0.39±0.02	0.66±0.04	*** P < 0.001
ADO	0.52±0.05	0.38±0.01	** P < 0.01
INO	1.68±0.19	2.08±0.11	P > 0.05
HX	1.87±0.26	5.58±0.56	*** P < 0.001

Cells were pre-incubated with [^{3}H]ADE for 30 min. Extracellular media was collected from co-cultures in the control condition after 30 min at 37^{o}C. Samples were analyzed for AN, ADO, INO and HX. Data are expressed as means (pmol/mg) ± SEM (n = 26-33). Data were analyzed by Student's T test; *** p < 0.001, ** p < 0.01.

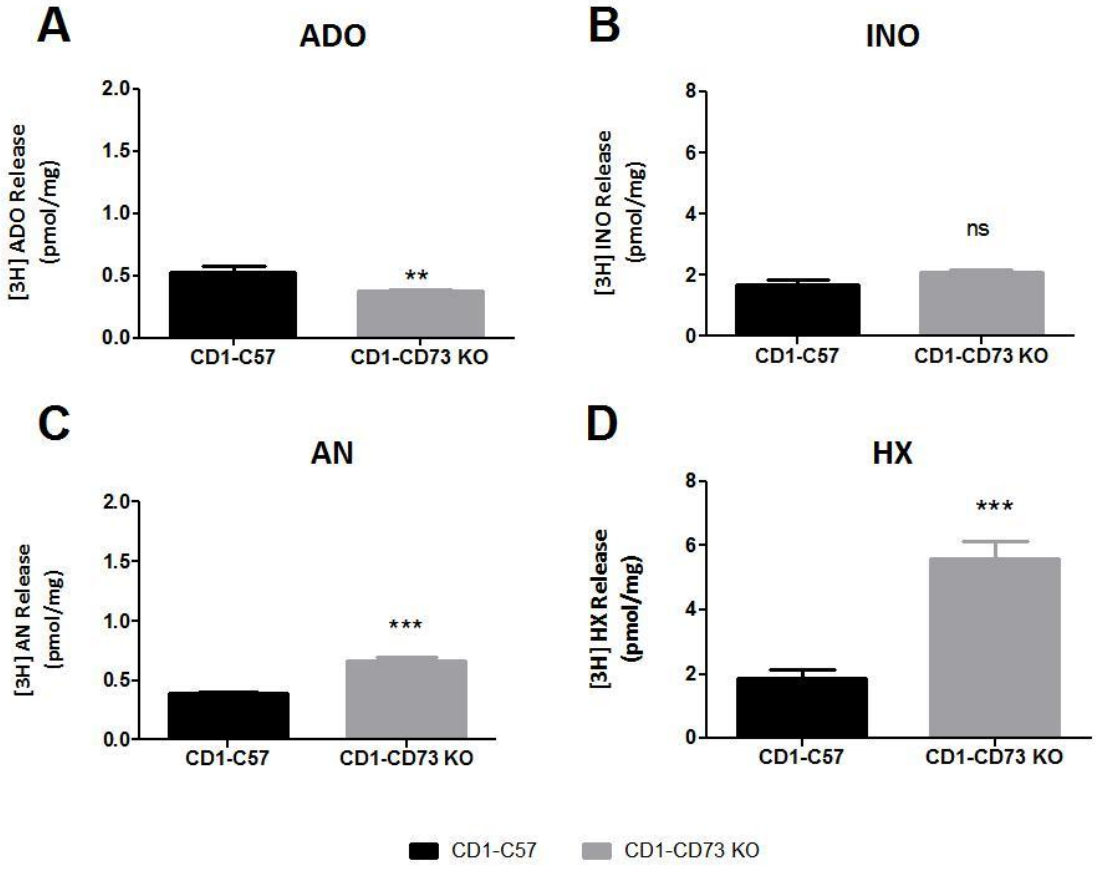

A
ADO
[3H] ADO Release (pmol/mg)
2.0
1.5
1.0
0.5
0.0
**
CD1-C57
CD1-CD73 KO
B
INO
[3H] INO Release (pmol/mg)
8
6
4
2
0
ns
CD1-C57
CD1-CD73 KO
C
AN
[3H] AN Release (pmol/mg)
2.0
1.5
1.0
0.5
0.0
***
CD1-C57
CD1-CD73 KO
D
HX
[3H] HX Release (pmol/mg)
8
6
4
2
0
***
CD1-C57
CD1-CD73 KO
CD1-C57
CD1-CD73 KO

Figure 4.9: The effect of NMDA-evoked release of A) ADO B) INO C) AN and D) HX from wt (CD1-C57) and eN deficient (CD1-CD73) co-cultures.

Cells were pre-incubated with [^{3}H] ADE for 30 min and then treated with 100 µM NMDA. Supernatants were collected after 30 min incubation at 37^{o}C. [^{3}H]Purines were separated by TLC and analyzed by scintillation spectroscopy. Data are expressed as mean ± SEM (n = 27-33). Data were analyzed by Student's T test; *** $p < 0.001$, ** $p < 0.01$.

Between the two cell culture types, there were no significant differences in the amount of ADO released when treated with DPR, AOPCP or NBMPR (Figure 4.10). In wt co-cultures, DPR inhibited NMDA-induced ADO release, whereas the eN inhibitor, AOPCP, and the selective ENT1 inhibitor NBMPR did not affect ADO levels. In eN deficient cultures, DPR, but not AOPCP or NBMPR, significantly decreased NMDA-evoked ADO release (Figure 4.10).

DPR showed a trend to decrease INO release from wt cultures; this was statistically significant in eN deficient cultures. AOPCP had no effect on INO release from either culture. NBMPR significantly decreased INO release from eN deficient cultures but not wt cultures. NMDA did not consistently increase INO release from co-cultures.

AN levels in wt or eN deficient cultures were unaffected by DPR, AOPCP or NBMPR. In addition, AN levels were not affected by NMDA. However, AN levels were consistently higher in eN deficient cultures than in wt cultures (Figure 4.11).

HX was the most abundant purine released by wt and eN deficient cultures, HX levels were not affected by NMDA, DPR, AOPCP or NBMPR in both CD1-C57bl6 and CD1-CD73 co-cultures (Figure 4.11).

Table 4.10: Extracellular [^{3}H]purine values from wt (CD1-C57) co-culture.

Treatment	AN	ADO	INO	HX
Control	0.39±0.05	0.13±0.02	0.87±0.16	2.30±0.56
DPR	0.43±0.04	0.12±0.01	0.27±0.02	2.82±0.39
NMDA	0.38±0.02	***0.46±0.09	*1.73±0.34	1.95±0.40
DPR + NMDA	0.39±0.04	+++0.14±0.02	+++0.32±0.05	1.72±0.25
Control	0.30±0.02	0.15±0.03	0.54±0.10	1.74±0.56
AOPCP	**0.46±0.04	0.13±0.01	0.65±0.11	2.11±0.61
NMDA	0.35±0.03	***0.52±0.08	1.34±0.30	1.48±0.45
AOPCP + NMDA	0.36±0.03	**0.44±0.09	1.86±0.64	1.74±0.59
Control	0.61±0.12	0.20±0.04	1.06±0.19	2.92 ±0.83
NBMPR	0.50±0.02	0.21±0.03	0.86±0.17	3.02±0.73
NMDA	0.47±0.02	**0.63±0.09	*2.22±0.29	2.46±0.51
NBMPR + NMDA	0.40±0.05	**0.62±0.10	1.74±0.31	2.04±0.46

Co-cultures were pre-treated with [^{3}H] ADE for 30 min. Then wt co-cultures were treated with 30 µM DPR, 50 µM AOPCP or 100nM NBMPR alone or in combination with 100 µM NMDA. The extracellular media was collected and analyzed for [^{3}H]purines. Data are expressed as means (pmol/mg) ± SEM (n = 6-12). Analysis was performed by one-way ANOVA and post-hoc analysis with Tukey's tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine; +++ $p < 0.001$ compared to NMDA treatment.

Table 4.11: Extracellular [^{3}H]purine values from eN deficient (CD1-CD73 KO) co-culture.

Treatment	AN	ADO	INO	HX
Control	0.81±0.10	0.16±0.01	1.59±0.19	5.70±0.98
DPR	0.88±0.11	0.11±0.02	***0.37±0.03	5.92±1.00
NMDA	0.70±0.06	***0.37±0.02	*2.17±0.21	5.81±0.91
DPR + NMDA	0.75±0.10	+++0.13±0.02	***0.52±.05	5.47±0.87
Control	0.73±0.08	0.14±0.02	1.42±0.26	6.03±0.27
AOPCP	0.71±0.06	0.10±0.01	1.37±0.26	5.67±1.21
NMDA	0.56±0.06	***0.35±0.02	1.85±0.20	5.51±1.19
AOPCP + NMDA	0.68±0.08	***0.32±0.03	1.74±0.17	5.22±1.08
Control	0.74±0.07	0.16±0.02	1.52±0.20	5.78±1.03
NBMPR	0.68±0.07	0.16±0.01	*0.95±0.12	5.38±1.02
NMDA	0.70±0.07	***0.40±0.02	*2.17±0.15	5.41±0.94
NBMPR + NMDA	0.78±0.09	***0.45±0.04	1.77±0.10	5.20±0.88

Co-cultures were pre-treated with [^{3}H] ADE for 30 min. Then eN co-cultures were treated with 30 µM DPR, 50 µM AOPCP or 100nM NBMPR alone or in combination with 100 µM NMDA. The extracellular media was collected and analyzed for [^{3}H]purines. Data are expressed as means (pmol/mg) ± SEM (n = 9-12). Analysis was performed by one-way ANOVA and post-hoc analysis with Tukey's tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to control level of purine; +++ $p < 0.001$ compared to NMDA treatment

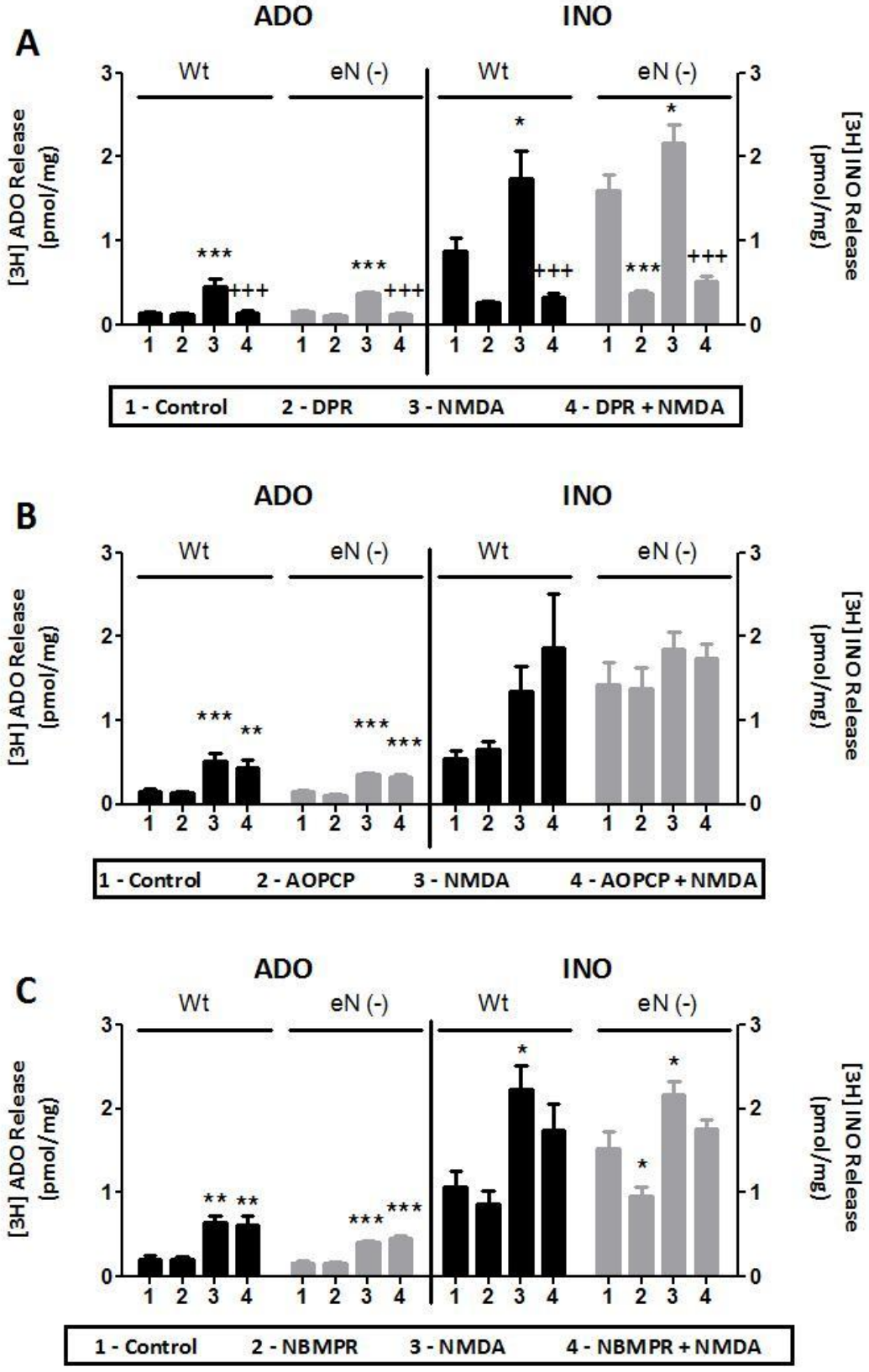
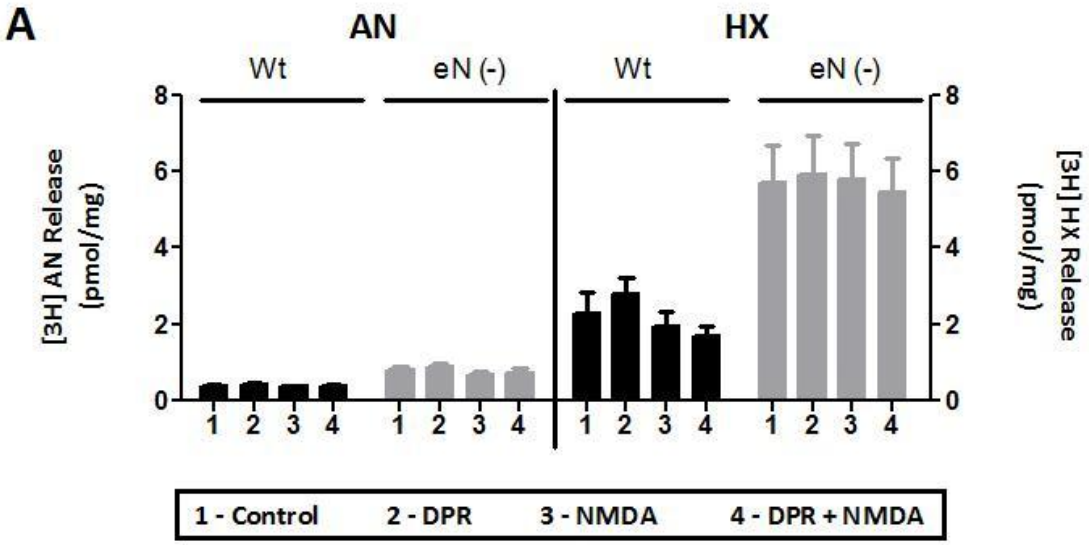
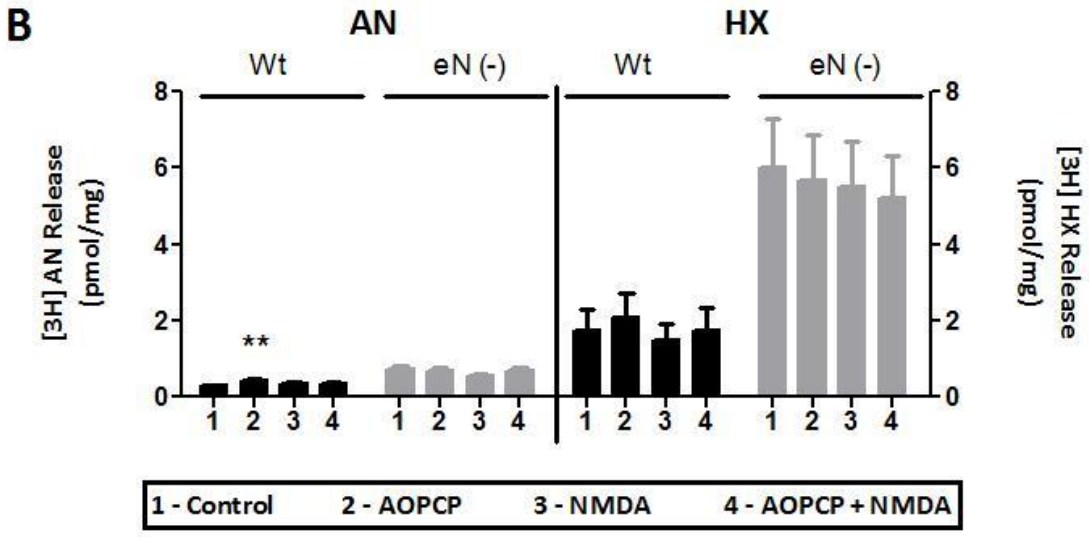

A
ADO
INO
Wt
eN (-)
Wt
eN (-)
[3H] ADO Release (pmol/mg)
[3H] INO Release (pmol/mg)
***
+++
*
1 - Control
2 - DPR
3 - NMDA
4 - DPR + NMDA
B
ADO
INO
**
1 - Control
2 - AOPCP
3 - NMDA
4 - AOPCP + NMDA
C
ADO
INO
1 - Control
2 - NBMPR
3 - NMDA
4 - NBMPR + NMDA

Figure 4.10: Comparison between ADO and INO release from wt (CD1-C57) and eN deficient (CD1-CD73 KO) co-cultures.

Co-cultures were incubated with [^{3}H]ADE then treated with A) DPR, B) AOPCP or C) NBMPR. Supernatants were collected after 30 min incubation at 37^{o}C. [^{3}H]Purines were separated by TLC and analyzed by scintillation spectroscopy. Statistical analysis between control and treatment groups for wt (CD1-C57) or eN deficient (CD1-CD73 KO) co-cultures was performed by one-way ANOVA and post hoc analysis with Tukey's tests. *** $P < 0.001$; ** $P < 0.05$, * $P < 0.01$ compared with wt or eN deficient co-culture controls. +++ $P < 0.001$ compared to wt or eN deficient NMDA treatment.


A
AN
HX
Wt
eN (-)
Wt
eN (-)
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
0
2
4
6
8
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
1 - Control
2 - DPR
3 - NMDA
4 - DPR + NMDA


B
AN
HX
Wt
eN (-)
Wt
eN (-)
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
**
0
2
4
6
8
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
1 - Control
2 - AOPCP
3 - NMDA
4 - AOPCP + NMDA

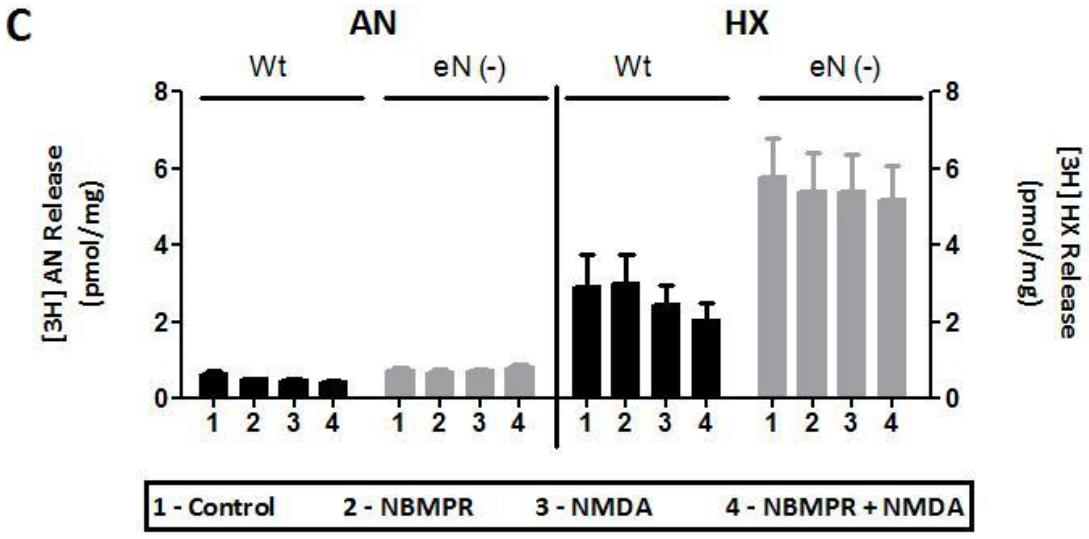

C
AN
HX
Wt
eN (-)
Wt
eN (-)
[3H] AN Release (pmol/mg)
[3H] HX Release (pmol/mg)
0
2
4
6
8
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
1 - Control
2 - NBMPR
3 - NMDA
4 - NBMPR + NMDA

Figure 4.11: Comparison between AN and HX release from wt (CD1-C57) and eN deficient (CD1-CD73 KO) co-cultures.

Co-cultures were incubated with [^{3}H]ADE then treated with A) DPR, B) AOPCP or C) NBMPR. Supernatants were collected after 30 min incubation at 37^{o}C. [^{3}H]Purines were separated by TLC and analyzed by scintillation spectroscopy. Statistical analysis between control and treatment groups for wt (CD1-C57) or eN deficient (CD1-CD73 KO) co-cultures was performed by one-way ANOVA and post hoc analysis with Tukey's tests. *** $P < 0.001$; ** $P < 0.05$, * $P < 0.01$ compared with wt or eN deficient co-culture controls. +++ $P < 0.001$ compared to wt or eN deficient NMDA treatment.

Chapter 5: Discussion

Brain ischemia arises from trauma, stroke or cardiac arrest. Disruption of oxygen and glucose supply to brain regions will lead to severe brain damage if blood flow is not reinstated. Stroke is one of the leading causes of disability and death; unfortunately few interventions are able to deal with the consequences of ischemic brain injury.

The research focus on neurons has been extensive; these cells are particularly sensitive to ischemic insult. Neuronal death is attributed to cumulative events such as depletion of ATP stores, intracellular acidification, generation of reactive oxygen species, loss of ion gradients and membrane depolarization resulting in excessive glutamatergic transmission (Choi and Rothman 1990) and other neurotransmitter release (Rossi et al.,2007).

Astrocytes on the other hand, have a more dynamic role integrating and communicating information. Astrocytes are less sensitive to ischemic damage. These cells contain glycogen and are able to maintain ATP levels longer than neurons. Astrocytes, therefore, maintain ionic gradients for longer in ischemic events and are less susceptible to glutamate excitotoxicity. Astrocytes are known to regulate synaptic activity through the release of glutamate, D-serine, ATP and ADO.

During ischemic conditions, ADO levels rise significantly (Parkinson et al., 2000; Phillis et al., 1996). Under these conditions, neurons become depolarized and lose their ability to maintain ionic gradients. Excitable neurons release the neurotransmitter glutamate which stimulates the NMDA receptor facilitating Ca^{2+} and Na^{+} influx. Excessive accumulation of intracellular Ca^{2+} can be toxic to cells. Calcium influx, together with Ca^{2+} released from internal stores, activates many proteases, lipases,

phosphatases and endonucleases that eventually cause cell injury and cell death from damage to vital cellular organelles. This 'excitotoxicity' by excessive glutamate activation of the NMDA receptor will lead to necrosis or activation of apoptotic pathways (Olney 1969, Orrenius et al., 2003).From previous *in vivo* and *in vitro* experiments, NMDA stimulation has been shown to produce ADO (Craig and White 1991; Lu et al., 2003). ADO released under pathophysiological conditions activates A_1 receptors, which affords a degree of neuroprotection by decreasing glutamate transmission, decreasing NMDA receptor activity, activating K^+ channels and reducing Ca^{2+} currents (Rudolphi et al., 1992a; de Mendonça et al., 1995).

ADO can be derived from intracellular or extracellular pathways through the metabolism of AN. Cytosolic or membrane bound nucleotidases breakdown AN to ADO. Bi-directional nucleoside transporters transport ADO across cell membranes. The excitatory and inhibitory actions of P1 and P2 receptors are dependent on extracellular concentrations of AN and ADO. While the actions of the receptors are well characterized, the cellular source contributing the sudden rise of ADO in pathophysiological conditions is still poorly characterized.

During an ischemic event, ENTs could potentially be important in the release or uptake of ADO. The activities of ENTs are determined by concentration of ADO produced by extracellular or intracellular pathways. To further study the role of ENTs in ischemia, mice with neuronal over-expression of ENT1 were produced (Parkinson et al., 2009). We hypothesized that Tg neurons with increased expression of hENT1, will release more ADO than wt neurons. A selective inhibitor for ENT1 is available; this is

NBMPR (also known as NBTI). Several other inhibitors are poorly selective for ENT subtypes, including DPR, which is used to inhibit both ENT1 and ENT2. The increased expression of hENT1 did not affect expression of ADO receptors or ADO metabolizing enzymes compared to wild type littermates. Interestingly, the Tg mice were more sensitive to the effects of ethanol and showed decreased response to caffeine (Parkinson et al., 2009).

At the cellular level, wt and Tg neurons were isolated and assessed for differences in release of the purines AN, ADO, INO and HX. In basal conditions, Tg neurons showed lower purine levels than wt but was not significantly different in ADO, INO and HX; INO was the most abundant purine in basal conditions and greater in wt than Tg neurons. Similarly, basal AN levels were significantly higher in wt neurons (Table 1). Lower extracellular purine levels in Tg neurons could be attributed uptake by ENTs.

NMDA treatment is a model for glutamate excitotoxicity *in vivo* and *in vitro* studies. NMDA-evoked ADO and INO release was observed in wt and Tg neurons, consistent with previous research using rat brain slices and rat neuronal cultures (Hoehn and White, 1989; Manzoni et al., 1994; Lu et al., 2003; Pedata et al., 1991). In comparison to wt neurons, Tg neurons showed greater NMDA-evoked [^{3}H]ADO and [^{3}H]INO release but [^{3}H]AN release was considerably less (Figure 4.1; Table 4.2). As well, there was significant inhibition in both wt and Tg neurons of [^{3}H]ADO and [^{3}H]INO release with non-selective ENT inhibitor, DPR (Zamzow et al., 2008b).

Since DPR inhibits both ENT1 and ENT2, we also used the selective ENT1 inhibitor NBMPR. From previous research with rat neurons we know that NBMPR has little effect on ADO release and ENT2 is the main transporter for ADO and INO (Zamzow et al., 2008b). In both wt and Tg neurons, we did not observe inhibition of NMDA-evoked [^{3}H]ADO or [^{3}H]INO release, using 100 nM, a concentration of NBMPR that is selective for ENT1. We expected in wt neurons for NBMPR to have little effect on ADO release. Although Tg neurons have increased expression of hENT1, we still did not see ENT1 inhibition with NBMPR. At low concentrations (below 1μM), NBMPR is a selective ENT1 inhibitor (K_d 0.1-10 nM) (Griffiths et al., 1997a). Hippocampal cell membranes isolated from hENT1 Tg mice have 15-fold increased abundance of [^{3}H]NBMPR binding sites, compared to membranes from wt littermates (Zhang et al., 2011). It is not clear why NBMPR did not inhibit ADO and INO release from Tg neurons, but it is possible that NMDA-evoked release occurs faster than the inhibitory effects of this low concentration (100 nM) of NBMPR with ENT1.

NMDA-evoked ADO release was not inhibited with eN inhibitor, AOPCP (Figure 4.2, Table 4.3 and 4.4), in contrast to the inhibition seen with DPR. The expression of eN on cortical neurons is low (Schoen et al., 1987; Parkinson et al., 2006); as expected, AOPCP had little effect on the production of NMDA-evoked ADO.

Similarly, Zhang et al., 2011 suggested that under ischemic events, neuronal ENT1 mediates increased ADO uptake rather than release. These experiments suggest that with hippocampal slices, the fEPSP slope and amplitude were increased in Tg slices vs. wt slices, as increased uptake of ADO decreased A_1 receptor activation. While this

study found that cortical neurons in culture release adenosine per se, the results of Zhang et al using hippocampal slices indicate neuronal uptake of ADO is greater than the release of ADO under ischemic conditions. This may indicate that the direct release of ADO from neurons is relatively minor relative to AN release from other cell types, such as astrocytes. Another source of ADO comes from release and extracellular metabolism of AN. ADO is metabolized to INO and then to its end product, HX, which occurs in astrocytes (Zamzow et al., 2008a). In basal conditions, [^{3}H]AN levels were higher in wt than Tg neurons, but levels of [^{3}H]HX were not significantly different. Tg neurons released [^{3}H]AN and [^{3}H]HX under NMDA-stimulation, which was not observed in wt neurons (Figure 4.3, Table 4.3 and 4.4). The extracellular levels of [^{3}H]AN and [^{3}H]HX in wt and Tg neurons were not affected by nucleoside transporter inhibition or eN inhibition. These experiments provide further evidence for neuronal release of ADO. Tg hENT1 neurons showed greater ADO release, suggesting intracellular formation and transporter mediated release.

Other studies have shown that astrocytes contribute to increasing levels of ADO in the CNS. The astrocytes release AN through various mechanisms, including vesicular release, connexin hemi-channels, pannexins, ATP-binding cassette transporters and anion channels (Pankratov et al., 2006; Dubyak 2006; Scemes et al. 2007). At present, the eN is the best characterized enzyme for the production of ADO extracellularly. In our experiments we used the CD73 KO mice, which lack this key enzyme. We hypothesized that the absence of eN or CD73 will significantly decrease ADO levels in CD73 KO astrocytes as compared to wild type in GD or OGD conditions.

In energy depleted conditions, such as of oxygen and/or glucose deprivation, astrocytes release purines (Parkinson et al., 2002; 2004). In the astrocyte cultures we were able to inhibit glucose utilization with 2-deoxy glucose (2DG), a competitive inhibitor of hexokinase, a key enzyme in glycolysis. Oxidative phosphorylation was reduced when astrocytes were in a humidified chamber containing 95% N_2 and 5% CO_2. We observed the greatest amount of ADO evoked from astrocytes occurred with inhibition of both glycolysis and oxidative phosphorylation (OGD) for 60 min (Parkinson and Xiong 2004). In addition, we included the ENT1 and ENT2 inhibitor, DPR, and the eN inhibitor AOPCP on the effect of purine release in ischemic-like conditions.

The CD73 KO mice exhibited low levels of ADO in colon, lung, heart, skeletal muscle, brain, liver, and kidney. These mice showed significant vascular leakage during hypoxia in all areas of the body (Thompson et al., 2004). In the ischemic brain, CD73 may also play a role in brain inflammation and immune function (Petrovic-Djergovic et al., 2012). CD73 KO tissues and astrocytes were confirmed of their phenotype (Figure 4.4 and 4.5). In both wt and CD73 KO astrocytes, there were no differences seen in the amount of total extracellular purines in basal, GD and OGD conditions (Figure 4.6). Upon closer inspection, there were significant differences observed with [^{3}H]ADO and [^{3}H]AN between wt and KO astrocytes. In line with previous reports [references], GD and OGD significantly increased [^{3}H]ADO release in wt astrocytes (Table 4.5 and 4.6). KO astrocytes had significantly reduced [^{3}H]ADO release in basal GD and OGD conditions (Figure 4.7; Table 4.5 and 4.7). DPR did not inhibit [^{3}H]ADO release in either wt or KO astrocytes. In some experiments, DPR increased levels of [^{3}H]ADO released by wt

astrocytes (Figure 4.7). These findings suggest that a significant amount of ADO is formed by the extracellular pathway and inhibition of ENTs reduces uptake into cells.

In wt astrocytes, extracellular levels of [^{3}H]ADO significantly dropped when eN was inhibited by AOPCP (Figure 4.7, Table 4.5 and 4.7). Although in wt cells [^{3}H]ADO was reduced by AOPCP, the levels did not equate to [^{3}H]ADO seen in KO astrocytes. A paper by Zimmermann (2009) suggested that other ectoenzymes present may also play a role in ADO formation in addition to eN such as prostatic acid phosphatases (PAP).

In astrocyte cultures, CD73 KO astrocytes tended to have higher levels of [^{3}H]AN in basal and GD conditions, although in OGD conditions [^{3}H]AN was significantly higher (Table 4.5). In wt and KO astrocytes, [^{3}H]AN levels were not affected by DPR. However inhibition with AOPCP increased [^{3}H]AN levels in wt astrocytes, while KO astrocytes were not affected (Table 4.6 and 4.7). We suspect that inhibition of eN or lack of eN generates build up of AN from decreased metabolism of AMP.

In both wt and KO astrocytes, [^{3}H] INO and [^{3}H]HX were the most abundant purines released and were not significantly different between the cell types (Table 4.5). The ENT1 and ENT2 inhibitor, DPR, was effective at decreasing [^{3}H]INO levels but not [^{3}H]AN, [^{3}H]ADO or [^{3}H]HX in basal, GD and OGD conditions in both wt and KO CD73 astrocytes (Figure 4.7 and 4.8, Table 4.6 and 4.7). Inhibition of nucleoside transporters prevented INO release from intracellular production. In ischemic conditions, INO has been shown to be neuroprotective (Haun et al. 1996). DPR and AOPCP had little effect on extracellular levels of [^{3}H]HX. KO astrocytes seemed to produce less [^{3}H]HX than wt astrocytes (Figure 4.8, Table 4.6 and 4.7). Previous reports suggest that astrocytes are

responsible for HX formation. INO released from neurons or astrocytes is taken up by astrocytes and metabolized to HX by PNP (Zamzow et al., 2008a). In summary, cortical astrocytes metabolize AMP to ADO by eN in the extracellular pathway. Astrocytes lacking expression or inhibition of eN produced little [^{3}H]ADO extracellularly.

So far in this work we showed in cell culture, neurons produce ADO by intracellular pathway and it is effluxed via ENTs. As well, astrocytes under GD or OGD conditions produce ADO from an extracellular pathway via eN. We hypothesized that eN is a major contributor for the production of ADO in the CNS, given the recent results Zhang et al., 2011 and further hypothesized that the absence of eN in co-cultures would produce less ADO compared to wild type cultures. Numerous studies done on brain slices or *in vivo* experiments have both cell types. Also eN is mainly located on astrocytes. Therefore the presence of both cell types will more accurately depict the relative pathways that are important in ischemic-like events. We established two types of cultures; wt co-cultures consisting of wt neurons (CD1) and wt astrocytes (C57bl6) and eN deficient co-cultures consisting of wt neurons and KO astrocytes (CD73 KO).

At basal conditions [^{3}H]HX was the most abundant purine in eN deficient co-cultures. Other purines [^{3}H]AN, ADO and INO were not statistically different from wt co-cultures (Table 4.8). We observed NMDA-evoked purine release from wt and eN deficient co-cultures. Overall, in both cultures NMDA-evoked significant increases of [^{3}H]AN and [^{3}H]HX in eN deficient cultures, but [^{3}H]ADO was significantly higher in wt co-cultures (Figure 4.9 and Table 4.9).

AN and HX were not affected by NMDA or DPR, AOPCP and NBMPR. In eN deficient co-cultures there was greater release of HX than in wt cultures (Figure 4.11, Table 4.10 and 4.11). With inhibition of both ENT1 and ENT2 there was a significant decrease of ADO and INO release evoked by NMDA (Figure 4.10, Table 4.10 and 4.11). No inhibition of NMDA-evoked ADO levels were observed in wt co-cultures with the selective ENT1 inhibitor NBMPR, although NBMPR showed a trend to decrease NMDA-evoked INO release (Figure 4.10, Table 4.10 and 4.11).

The eN inhibitor, AOPCP did not affect [^{3}H]ADO release from eN deficient co-cultures, which was expected because of the lack of eN on KO CD73 astrocytes. In wt co-cultures AOPCP did decrease ADO levels; however this was only a modest decrease and was not statistically significant (Figure 4.10, Table 4.10 and 4.11). This suggests that eN does not have a prominent role in ADO formation. This was in contrast to our hypothesis, and in contrast to other reports that showed AOPCP to be effective at decreasing ADO in rat neuron-astrocyte co-culture conditions (Zamzow et al., 2008b). Conceivably, the expression of eN in co-cultures is low, as other reports have shown that eN expression is induced in ischemic conditions with an ensuing rapid rise of extracellular ADO (Braun et al., 1998). The increase of eN expression is correlated with a change in transcription of genes, neuronal cell damage or death (Sweeney et al., 1995). The expression levels of eN were not measured in co-cultures, although the expression in wt astrocyte cultures was demonstrated (Figure 4.4).

Although the primary objective of these experiments was to examine ADO release in co-cultures we also looked at INO levels. The results showed INO was evoked

by NMDA stimulation and this release was inhibited by DPR (and NBMPR). It was previously hypothesized that ADO exerts neuroprotection by activating A_1 and A_2 receptors on astrocytes (Van Calker et al., 1979). Activation of A_1 decreases adenylyl cyclase whereas activation of the A_2 receptors results in activation of adenylyl cyclase in astrocyte cultures (Van Calker et al., 1979; Hölsi et al., 1987; Woods et al., 1988, Murphy et al., 1991). In astrocyte cultures, the neuroprotective effect of ADO was not attributed to ADO receptor stimulation but by INO (Haun et al., 1996). In the co-cultures, if INO does mediate protection in astrocytes preventing ischemic-induced expression of eN in astrocytes, this could be an explanation for why we did not observe the effects of AOPCP in the co-cultures.

INO does not have a specific receptor to act on, but weakly binds to ADO receptors (Linden 2001). INO is known to weakly bind to benzodiazepine receptors (K_i > 1.0 mM; Skolnick et al., 1980). The mechanism of action of INO may be due to intracellular secondary messenger signaling pathway. In cultured sympathetic neurons, catecholamine production was stimulated with INO and this effect was blocked by DPR (Zurn and Do 1988). Activation of guanylyl cyclase by sodium nitroprusside potentiated the effect of INO on catecholamine production which suggests that cGMP may play a role in INO-mediated astrocyte protection (Zurn 1991).

Another report suggested that astrocyte cultures could be pre-conditioned to OGD conditions if there was prior exposure to sub-lethal OGD conditions (Iwabuchi and Kawahara 2009). This phenomenon could be attributed to ATP-P2Y receptor signaling resulting in PLC-IP_3 pathways and subsequent Ca^{2+} release from endoplasmic reticulum,

promoting spontaneous Ca^{2+} oscillations in astrocytes. Although our co-culture experiments did not use OGD conditions, this potential relationship could also affect the transcription of eN expression in astrocytes under ischemia like conditions.

Interestingly, in the eN deficient cultures [^{3}H]AN release were higher than in wt cultures. From *in vivo* or *in vitro* experiments, it's been shown that the neuron-astrocyte interaction converts AN to ADO. Despite the absence of eN on astrocytes, ADO levels still increased with NMDA stimulation. Therefore, the only other source of ADO should come from neurons. Although astrocytes are known to be important in brain homeostasis and influence neuronal activity, recent data suggest they are also important in pathophysiological conditions. A recent report by Eduardo Martin suggested that astrocytes may be relevant in hypoxia/ischemic conditions in the brain as they release a variety of gliotransmitters, one of which could be ADO (Martin et al., 2007).

Literature on the intracellular or extracellular origin of ADO is extensive and yet consensus on ADO release or extracellular metabolism to ADO has not been reached. There are many neuronal preparations and methods to stimulate ADO release which could account for these differences of intracellular or extracellular origin of ADO. Numerous *in vivo* and *in vitro* studies suggest that AN released are subsequently converted to ADO by eN. In cell culture studies both neurons and astrocytes release significant amounts of ADO in ischemia-like conditions (Parkinson et al., 2002, 2005; Parkinson and Xiong 2004). In these conditions, monocultures of neurons directly release ADO, where as astrocytes release AN and subsequent conversion to ADO by eN

(Zamzow et al., 2008). Inhibition of eN in NMDA-evoked ADO conditions reduced ADO release (Zamzow et al., 2008). Alternatively in rat cortical neurons, eN inhibition did not affect NMDA-evoked ADO release (Lu et al., 2003).

Electrophysiological approaches using hippocampal slices noted ATP is rapidly converted to ADO which then activates A_1 receptor and increases K^+ conductance on post-synaptic neurons. As well, inhibition of eN determined that conversion of AMP is the rate-limiting step in the formation of ADO (Dunwiddie et al., 1997; Cunha et al., 1998). From this perspective, production of ADO from eN may be inhibited by the high release of ATP or ADP that inhibit eN; when levels of ATP and ADP are lower, the extracellular production of ADO can occur (James and Richardson 1993).

Recently it was found that neurons that are actively stimulated mediate synaptic depression by the release of ADO from ENTs. Selective astrocytic activation of ATP-mediated vesicle release by Ca^{2+} photolysis did not evoke synaptic depression, whereas selective neuronal firing mediates the release of ADO and synaptic depression. The authors did not discount that ATP is still released into the environment, but the amount of ADO is not sufficient to activate A_1 receptors. They concluded that under conditions of excessive neuronal firing, adenosine is released from neurons as a negative feedback mechanism to inhibit excessive excitatory events (Lovatt et al., 2011).

The data gathered from these experiments confirmed that *in vitro* conditions neurons effluxed ADO via nucleoside transporters. The overall activity of hENT1 in Tg neurons mediates nucleoside efflux as opposed to nucleoside uptake (Zhang et al., 2011). NMDA-evoked ADO release was observed in these neurons. In monocultures of

KO CD73 astrocytes, eN deficient cells did not produce ADO from AMP metabolism. We confirmed that in wt astrocytes, inhibition of eN blocked the release of ADO and nucleoside transporter inhibition had little effect. Thus the extracellular pathway is prominent in astrocytes. Previous reports suggested that AN released to the environment is metabolized to ADO by eN. Thus eN is an important mediator under these conditions. When we cultured both neurons and astrocytes together we expected to see inhibition of NMDA-evoked ADO release with AOPCP, however no significant inhibition was observed. In eN deficient co-cultures, wt neurons produced ADO intracellularly, which was effluxed by nucleoside transporters. From the current data it is not clear if inhibition of eN is a contributor to rising ADO levels in ischemic conditions. Although other factors such as up-regulation of eN expression during ischemic events or astrocyte mediated ADO release could have affected extracellular ADO concentrations in co-cultures.

ADO has numerous receptor-mediated effects in the CNS affecting a wide range of physiological and disease states. ADO is a neuromodulator of neuronal synaptic transmission and is neuroprotective in ischemic conditions. ADO is released from a number of cell types in the brain. There is conflict in elucidating the cellular contributions of the enzymes and transporters responsible for ADO formation and metabolism. The work in this thesis brings up new questions on the role of eN in ADO formation in the CNS.

Chapter 6: References

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