The Effects of Perturbations to Adenosine Receptor Signaling in Mice and Newborn Rats Exposed to Hypoxic and Ischemic Conditions

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Adenosine is a purine nucleoside which plays an important role in many biochemical processes. In addition, it controls many central nervous system (CNS) functions both under physiological and pathophysiological conditions. Adenosine acts through four adenosine receptors (AR), of which adenosine 1 receptors (A_1R) and adenosine 2A receptors ($A_{2A}R$) are of particular importance in CNS. This thesis aimed to investigate adenosine`s role in two major hypoxic conditions of early and late life of humans by changing its receptor functions using receptor antagonists. These two conditions were stroke, a problem in adults/elderly and apnea, a problem of early newborns.

Four studies were conducted. In the first study, we used intracortical (IC) endothelin-1 (ET-1) injection in mice to induce a cortical stroke. Using magnetic resonance imaging (MRI), we found that stroke volume was increased in transgenic (Tg) mice with over-expression of the adenosine transport protein equilibrative nucleoside transporter 1 (ENT1). Caffeine, an AR antagonist neutralized this difference.

In the second study, we used ET-1 to induce cortical stroke in mice and used the selective A_1R antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Both MRI and histopathology were used to determine stroke volume. We found a greater stroke size in Tg mice treated with the A_1R antagonist.

In the third and fourth studies, the effect of antenatal caffeine on ontogeny of ARs and respiratory response to intermittent hypoxia was examined. Antenatal exposure to high dose caffeine caused a significant increase in A_1Rs in the striatum on postnatal 1^{st} day (P0). Also, high dose caffeine exposure caused a significant increase in gene expression of A_1R and $A_{2A}Rs$ in the brain stem (BS). These changes disappeared by postnatal 7^{th} day (P6). There were several

detrimental effects of high dose caffeine on pregnancy outcome but prenatal exposure to high dose caffeine did not change the hypoxic respiratory response of newborn rats on P6.

We conclude that AR antagonists enhance ET-1 induced stroke injury in mice, however we did not observe a detrimental effect of antenatal caffeine on postnatal respiratory responses to transient hypoxia. Further research is needed to uncover the mechanisms by which AR antagonists affect responses to hypoxic conditions.

ACKNOWLEDGMENTS

*I would cordially thank to **Dr. Fiona Parkinson**, for numerous reasons. First of all, she has accepted me under her supervision as a student. She always treated me with a great patience and made an incredible empathy for understanding my efforts to manage my clinical works and PhD studentship together. She supported me during my transition from MSc to PhD. She always responded to me on time even I knew she was very busy. Therefore, as a student in mid 40s, I will feel very lucky forever for being a student for Dr. Parkinson.*

I would also thank to my advisory committee members

***Dr. Benedict Albensi**, for his very valuable advice and for teaching research trial design.*

***Dr. Marc DelBigio**, for his very valuable contributions on my studies and opening his laboratory for histopathological part of my research project. He also taught me how to measure cerebral damage on software.*

***Dr. Shyamala Dakshinamurti**, for her guidance for my application to the graduate program and her comments on my research projects. She is also a very dedicated clinician with whom working together in Neonatology is always a pleasure.*

***Dr. Donald Miller**, for his very valuable advice which always provided me with self-confidence.*

***Dr. Paul Fernyhough**, for his mentorship as a graduate chair and recommendations during my education.*

***Dr. Donald Smyth**, for his excellent teaching for the first year of my studentship. I should also note very important contribution of **Dr.Gordon Glazner** and **Dr. David Eisenstat** for my enhanced understanding of neuroscience; **Dr. Frank Burczynski** and **Dr. Depeng Jiang** for my improved understanding of pharmacokinetics and biostatistics. Also to **Dr. Daniel Sitar** for his*

guidance for my presentations. ***Dr. Andrew Halayko*** *and* ***Ms. Sujata Basu*** *for their support in respiratory physiology study.* ***Dr. Ratna Bose*** *should also be mentioned here for her support and kind encouragement during my studentship.*

My colleagues in our laboratory, ***Wei Xiong, Dali Zhang, Stephanie Chu, and Chao Sun*** *deserve a million thanks for their wonderful collaboration. Dali was also my first teacher who showed me how to establish a stroke by using endothelin. It was always a great pleasure to share the lab with them.*

In addition, I would like to thank to our former and current office staff ***Ms. Angie Ekosky, Ms. Karen Donald*** *and* ***Ms. Raquel Nowlin*** *in the Department of Pharmacology and Therapeutics who maintained my applications and processes on time.*

Finally, I cannot imagine this PhD would be possible without support of my colleagues in the Division of Neonatology at Faculty of Medicine especially to ***Dr. Ruben Alvaro***, ***Dr. Molly Seshi and Ganesh Srinivasan***. *Here, I should also state valuable comments of* ***Dr. Shabih Hasan*** *from University of Calgary for my thesis project. And thank you all in both departments for everything you made for my PhD education.*

This thesis is dedicated to my parents and to my wife.

To memory of my mother, Emine Soylu, who always motivated for my education, and my father, Mustafa Soylu, who passed on a love of reading and instilled the importance of hard work. This thesis would be incomplete without my wife's caring support of me and my ten children during my clinical and research work.

TABLE OF CONTENTS

LIST OF THE TABLES

Chapter IV

LIST OF THE FIGURES

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

LIST OF COPYRIGHTED MATERIALS

1. Figure 1 in Chapter I was originally published in Chu, S., W. Xiong, D. Zhang, H. Soylu, C. Sun, B.C. Albensi and F.E. Parkinson. 2013. Regulation of adenosine levels during cerebral ischemia. Acta Pharmacol Sin. 34:60-66. It is an open access article and authors retain copyright.

2. A version of Chapter II has been published as Soylu, H., D. Zhang, R. Buist, M. Martin, B.C. Albensi and F.E. Parkinson. 2012. Intracortical injection of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter. Exp. Transl. Stroke Med. 4:4. It is an open access article subject to Creative Commons Attribution (CC-BY) license.

LIST OF ABBREVIATIONS

A_1R	adenosine 1 receptors
$A_{2A}R$	adenosine 2A receptors
$A_{2B}R$	adenosine 2B receptors
A_3R	adenosine 3 receptors
AC	adenylyl cyclase
ADA	adenosine deaminase
AK	adenosine kinase
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
AOP	apnea of prematurity
AR	adenosine receptors
ATP	adenosine triphosphate
BBB	blood-brain barrier
BS	brain stem
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CC	caffeine-caffeine
Cg	caffeine group
CI	confidence interval
CMV	corrected minute volume
cN	cytosolic 5'-nucleotidase

CNS	central nervous system
CNT	concentrative nucleoside transporter
CS	caffeine-saline
CTV	corrected tidal volume
DMSO	dimethyl sulfoxide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
eN	ecto-5′-nucleotidase
ENT	equilibrative nucleoside transporter
ENT1	equilibrative nucleoside transporter 1
ET-1	endothelin-1
g	gram
GABA	γ-amino butyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hours
H&E	hematoxylin and eosin stain
hENT1	human equilibrative nucleoside transporter 1
hENT2	human equilibrative nucleoside transporter 2
HVD	hypoxic ventilatory decline
HVR	hypoxic ventilatory response
IC	intracortical
IP	intraperitoneal
IS	intrastriatal
kg	kilogram

LBW	low birth weight
l	liter
MCA	middle cerebral artery
µg	microgram
µl	microliter
µM	micromolar
mg	milligram
min	minutes
ml	milliliter
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MV	minute volume
NO	nitric oxide
OGD	oxygen glucose deprivation
OR	odds ratio
p	calculated probability
P0	postnatal 1st day
P6	postnatal 7th day
pmol	picomol
RARE	rapid acquisition refocused echo
RIPA	radioimmunoprecipitation assay
rt-PCR	real time polymerase chain reaction
s	seconds

SAH	S-adenosylhomocysteine
SD	standard deviation
SPSS	statistical package for the social sciences
Tg	transgenic
TV	tidal volume
WB	western blot
WC	water-caffeine
Wg	water group
WS	water-saline
Wt	wild type

Chapter I:

Introduction and Review of Adenosine

1. History of Adenosine

The history of adenosine is also partly a history of adenosine triphosphate (ATP). In 1929, Drury and Szent-Gyorgyi demonstrated that a heart muscle extract that contained adenylic acid decreased heart rate, decreased blood pressure and dilated coronary arteries (Drury and Szent-Gyorgyi, 1929). Despite the current well known effects of adenosine on the other systems, the first evidence of adenosine-related effects was heart related. In the 1970s, adenosine's role in CNS functions became an evolving research area in neuroscience. Sattin and Rall (1970) reported that adenosine increases cyclic adenosine monophosphate (cAMP) in the brain and adenosine's effect is prevented by theophylline, known as a phosphodiesterase (PDE) inhibitor. Two years later Burnstock (1972) started to use the term "purinergic" for ATP related neurotransmission. Actually, Burnstock was one of the pioneers in this area hypothesising that neurons can use purines as transmitters; however he was criticized by others and his theory was considered "a theory without any evidence" for a long time. However, the same author defined purinergic receptors in 1976 (Burnstock, 1976). One year later, adenine nucleotides were found to be modulators of noradrenaline release from sympathetic nerves supplying veins (Verhaeghe *et al.*, 1977). Finally, a year later, van Calker *et al.* (1978) showed ARs in brain cells and they were classified as A_1R (inhibitory) and A_2R (stimulatory). Later, adenosine 2B receptors ($A_{2B}R$) and adenosine 3 receptors (A_3R) were added to the nomenclature. Since 1980s, ARs have been studied more extensively into their functions in animal models and in human diseases.

2. Adenosine Synthesis and Metabolism

2.1 Intracellular source of adenosine.

Adenosine release from synapses does not follow nerve firing as with classical neurotransmitters (Jacobson and Gao, 2009). It is commonly thought that adenosine is formed intracellularly as a consequence of ATP utilization or extracellularly by a cascade of hydrolase enzymes to dephosphorylate ATP to adenosine (Chu *et al.* 2014). Physiologically, intracellular adenosine can be formed by either dephosphorylation of adenosine monophosphate (AMP) by cytosolic 5'-nucleotidase (cN), or, alternatively, by hydrolysis of S-adenosylhomocysteine (SAH) (Boison *et al.*, 2010). Since intracellular ATP levels are up to 100,000-fold greater than adenosine, any increase in energy requirements in neurons, such as seizures or ischemia, has the potential to increase the conversion of intracellular ATP to adenosine. This can lead to a dramatic increase in the intracellular concentration of adenosine, since a 1% conversion of ATP to adenosine through AMP results in an approximate 100-fold increase in intracellular adenosine concentration (Fredholm, 2005; Dunwiddie and Masino, 2001). The existence of a substrate cycle between AMP and adenosine, with the opposite activities of 5'-nucleotidase and adenosine kinase (AK), introduces a further amplification checkpoint to regulate the formation of intracellular adenosine upon changes in the concentration of intracellular AMP (Cunha, 2001). However, this adenosine which is present in a higher concentration inside than outside the cell, does not freely diffuse across the cell membrane and requires nucleoside transporters, such as the equilibrative nucleoside transporter (ENT), which works bidirectionally (Jacobson, 2009). It was also reported that the pathways for adenosine production differ between neurons and astrocytes. In general, neurons produce adenosine *per se*, whereas astrocytes convert extracellular adenine nucleotides to adenosine (Parkinson and Xiong, 2004). Astrocytes also serve as regulatory cells

for removal of excessive adenosine from the environment by metabolising it to inosine and hypoxanthine (Zamzow *et al.*, 2008). By this mechanism neurons could be considered main source of adenosine but astrocytes can also release smaller quantities of adenosine. However, some other studies from our lab showed that astrocytes could also be a main source of adenosine production under oxygen glucose deprivation (OGD) through ecto-5′-nucleotidase (eN) enzyme (Chu *et al.*, 2014).

2.2 Extracellular source of adenosine

Adenosine is also formed extracellularly from ATP or other adenine nucleotides that are released from cells to serve signaling functions through the purinergic P2X and P2Y receptor families (Parkinson, 2009; Boison *et al.*, 2010). The level of extracellular adenosine may be as low as 20 nM in the resting brain and as high as 100 micromolar (μM) in severe ischemic conditions in the brain or elsewhere in the body (Jacobson and Gao, 2009). ATP is released from neurons in synaptic vesicles together with other neurotransmitters (Dunwiddie and Masino, 2001; Latini *et al.*, 1995). In addition, ATP release and conversion of AMP to adenosine through eN by astrocytes are well documented and it provides another source of extracellular adenosine (Parkinson and Xiong, 2004, Chu *et al.*, 2014).

2.3 Adenosine fate

Adenosine clearance shows some differences from other neurotransmitters (Jacobson and Gao, 2009). Adenosine can either be converted into inosine by deamination through adenosine deaminase (ADA) or be phosphorylated into AMP via AK. Considering the relative Michaelis-Menton constant (Km) values, AK is important at low levels of intracellular adenosine and is

considered to be the primary route of adenosine metabolism, whereas ADA is more relevant at high concentrations of adenosine (Boison *et al*., 2010). Recent findings indicate that extracellular levels of adenosine, and consequently the levels close to synapses, are largely regulated by astrocytes (Fredholm *et al*., 2005; Boison *et al*., 2010; Zamzow *et al*., 2008a,b). Another enzyme of importance is S-adenosylhomocysteine hydrolase. This enzyme sets the equilibrium between S-adenosylhomocysteine and adenosine + L-homocysteine. When the level of L-homocysteine is low, this enzyme serves to generate adenosine (Fredholm *et al.,* 1999) (Figure 1).

3. Adenosine receptors

As a neuromodulator, adenosine acts through a family of metabotropic receptors which have been designated A_1, A_{2A}, A_{2B} and A_3 (Varma *et al.*, 2002, Fredholm *et al.*, 2005). These four ARs are also known as P_1 purinoceptors (Fredholm *et al.*, 2001). Using G-protein-coupled mechanisms, that not only lead to changes in second-messenger levels but also to the modulation of ion channels (such as Ca^{2+} and K^+ channels), adenosine modulates neuronal activity pre-synaptically by inhibiting or facilitating transmitter release, post-synaptically by affecting the actions of other neurotransmitters, and non-synaptically by hyperpolarizing or depolarizing neurons (Sebastião and Ribeiro, 2009).

A_1Rs, are located both pre- and post-synaptically throughout the brain, including cerebral cortex, striatum, hippocampus and cerebellum (Gessi *et al.*, 2011). The pre-synaptic receptors activate Gi/o proteins to inhibit Ca^{2+} channel activity (Cunha, 2001). The post-synaptic A_1Rs couple via Gi/o proteins to K^+ channels to hyperpolarize neurons and inhibit action potential propagation (Fredholm *et al.*, 2005; Dunwiddie and Masino, 2001; von Lubitz, 1999). In general

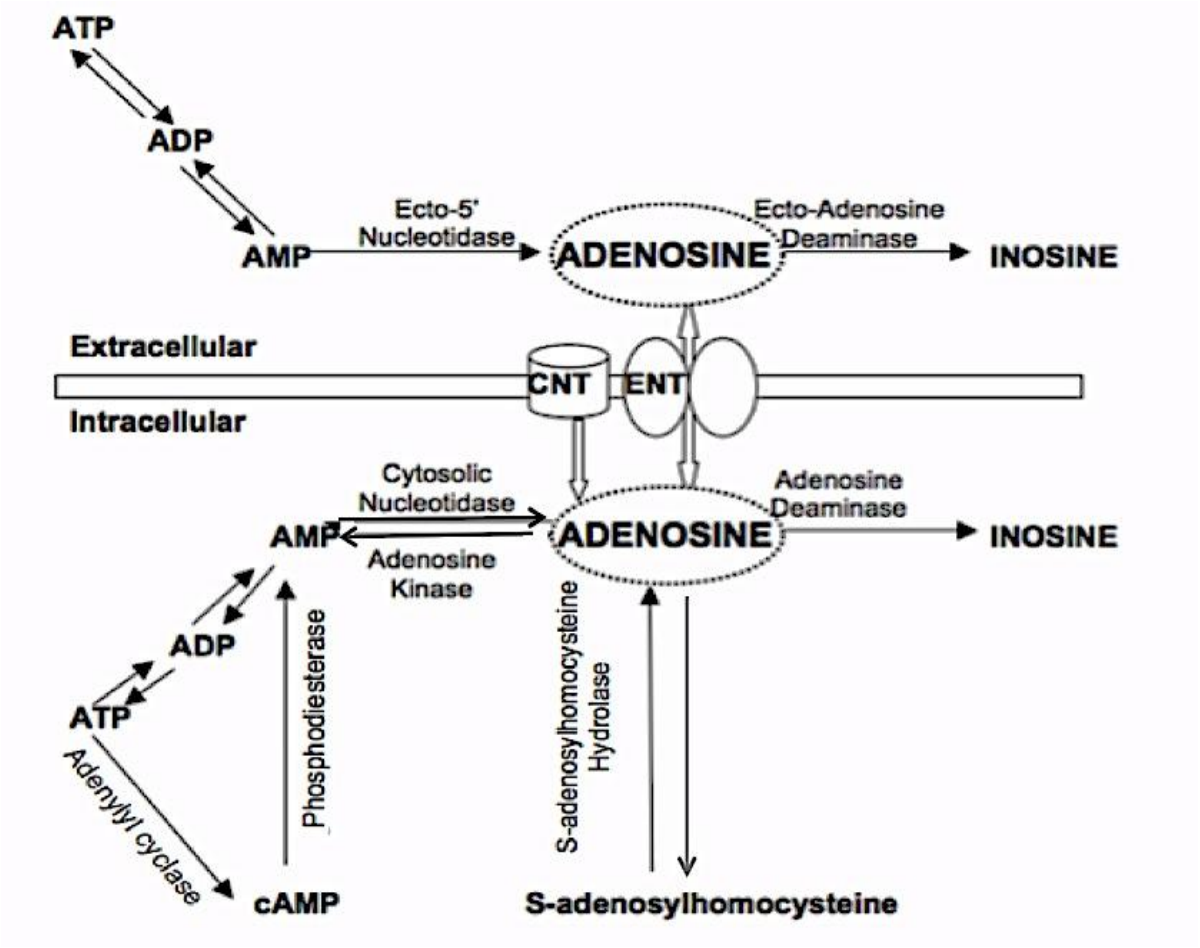


Figure 1. Pathways for adenosine formation and metabolism. Intracellularly, adenosine is primarily formed by dephosphorylation of ATP, although cAMP and SAH are alternative precursors for adenosine formation. Extracellularly, adenosine is thought to be a product of ATP that is metabolized by a series of ecto-enzymes. Nucleoside transport processes mediate cellular uptake or release of adenosine and other nucleosides, including inosine. Concentrative nucleoside transporter (CNT) and ENT are illustrated (Chu *et al.,* 2013).

A_1R activation has neuroprotective effects and can produce sedation, antinociception, anticonvulsant effects, bradycardia, vasoconstriction, bronchoconstriction, antidiuresis and decreased glomerular filtration, and an increase in insulin sensitivity (Ribeiro and Sebastião, 2010). Use of A_1R agonists in experimental settings results in decreased striatal lesion in Huntington's disease models, enhanced spatial learning and memory in stroke, decreased anxiety and decreased pain (Ribeiro *et al.,* 2003).

$A_{2A}Rs$ are described as stimulatory, since they couple to stimulatory G proteins, increase cAMP formation and can enhance neurotransmitter release through P-type calcium channels (Gessi *et al.*, 2011; Cunha, 2001). $A_{2A}Rs$ are highly expressed in striatum, nucleus accumbens and olfactory tubercle where they co-localize with dopamine D2 receptors (Stone and Ceruti, 2009). In contrast to A_1Rs, activation of $A_{2A}Rs$, can promote cell injury (Fredholm *et al.,* 2001). Activation of $A_{2A}Rs$ causes vasodilation, bronchodilation, central respiratory depression and peripheral respiratory stimulation, platelet inhibition, decreased locomotor activity and immunosuppression (Ribeiro and Sebastião, 2010). In CNS, it is mostly accepted that $A_{2A}Rs$ blockade or inactivation results in neuroprotection, thus $A_{2A}Rs$ work against A_1Rs (Fredholm *et al.,* 2005, Silva *et al.,* 2007, Cunha, 2005). However, a recent review stated that $A_{2A}R$ agonists may have a beneficial role in brain ischemia since it can cause vasodilation and increase expression and release of neurotrophic factors (Pedata *et al.,* 2015).

$A_{2B}Rs$ have been studied less extensively than the others. Our current knowledge shows that $A_{2B}Rs$ are expressed at low levels in almost all tissues including brain and spinal cord and have low affinity for adenosine (Stone *et al*., 2009). $A_{2B}R$ have the lowest affinity for adenosine among all ARs (Pedata *et al.,* 2015, Borea *et al.,* 2016). The other issue for $A_{2B}Rs$ is the relative lack of agonists and antagonists compared to other ARs (Muller and Jacobson, 2011). Current

literature suggests that A_{2B}Rs mostly play a detrimental role on neuronal cells and A_{2B}Rs antagonism might be protective for ischemic brain damage (Melani *et al.,* 2014).

The levels of A_3Rs in the brain are low, but higher in sheep and humans than in rodents (Salvatore *et al.*, 2000). Through the development of selective A_3R agonists, the putative pathophysiological roles of this receptor have become clear and it has been demonstrated that they affect cell survival, by promoting cell protection or cell death, depending upon the cell type and/or agonist concentration (Trincavelli *et al.,* 2002). A_1Rs and A_3Rs both inhibit AC activity (Borea *et al.,* 2009). Thus, A_{3A}R are able to reduce excitotoxicity and promote neuroprotection in neurons after ischemic damage (Borea *et al.,* 2014). However, the effect of A_3Ractivation depends on the severity and duration of the event (Pedata *et al.,* 2015). Stimulation of A_3Rs during a brief period of ischemia might exert A_1R like protective effect; however in prolonged ischemic conditions A_3R stimulation switches from protective to dangerous because of an inhibitory interaction with A_1R stimulation (Bjorklund *et al.*, 2008; Pedata *et al.,* 2015).

In sum, the combined activity of A_1R, A_{2A}R, andA_3R tightly tunes glutamatergic synaptic activity, modulating AMPA receptor activity and affecting many CNS related conditions (Di Angelantonio *et al.,* 2015). Some of these conditions are stroke, Huntington's disease, Parkinson's disease, schizophrenia, epilepsy and pain (Fredholm *et al.,* 2005).

4. Adenosine transporters (Nucleoside transporters)

Nucleosides and nucleoside analogs are hydrophilic molecules and require transporters to enter or exit cells (Gray *et al.,* 2004). Nucleoside transporters facilitate cellular uptake and/or efflux of various nucleosides and nucleobases. Nucleoside transporters play an important role in initiating or terminating adenosine signaling by delivering it to or removing it from extracellular

space, thus, controlling its availability for cell-surface adenosine receptors (Lu *et al.*, 2004). The degree and duration of activation of adenosine receptors by adenosine is heavily influenced by nucleoside transporters located on the plasma membrane of all cells.

Two families of nucleoside transporters have been identified: equilibrative and concentrative. These are coded for by the solute carrier gene families SLC29A and SLC28A, respectively (Gray *et al.*, 2004, Baldwin *et al.*, 2004). ENTs are bidirectional and sodium-independent, in contrast to CNTs, which are sodium-dependent and couple nucleoside influx to sodium influx (Gray *et al.*, 2004, Baldwin *et al.*, 2004). In the CNS, ENTs appear to be dominant (Thorn *et al.*, 1996).

ENTs carry nucleosides across cell membranes in either direction according to their concentration gradients (Anderson *et al.*, 1999). While four ENTs (ENT1, ENT2, ENT3 and ENT4) have been identified to date, only ENT1 and ENT2 appear to play an important role in regulating brain adenosine concentrations (Baldwin *et al.*, 2004; Young *et al.*, 2008). Anderson *et al.* (1999) reported that ENT1 messenger ribonucleic acid (mRNA) is present in several neuron populations in hippocampus, cortex, striatum, and cerebellum in rat brain. Another study from human brain showed that hENT1 was abundant in the frontal and parietal cortex, and it was of moderate abundance in the temporal and occipital cortex, thalamus, midbrain, caudate nucleus, putamen and globus pallidus (Jennings *et al*., 2001). In contrast, human equilibrative nucleoside transporter 2 (hENT2) was concentrated in the cerebellum and brainstem regions, particularly the pons and midbrain and low abundance was observed in cerebral cortex, hippocampus and basal ganglia (Jennings *et al.*, 2001).

ENTs can mediate influx or efflux of nucleosides depending on the relative substrate concentrations across the membrane (Molina-Arcas *et al.*, 2008). By altering the levels of

adenosine available to interact with cell–surface receptors, ENT1 and ENT2 have profound effects on the ability of adenosine to modulate neurotransmission, vascular tone and other physiological events (Jennings *et al.,* 2001; Baldwin *et al.,* 2004). The results from previous studies have illustrated that ENTs are important determinants of sensitivity to, and toxicity from, nucleosides and other related drugs, and might be a potential target for therapeutic strategies (Young *et al.,* 2008).

4.1 Consequences of blockade or over expression of ENT1

In the Parkinson laboratory, transgenic (Tg) mice with neuronal expression of hENT1 in a CD1 mouse genetic background were created and used to examine whether, and under what conditions, neurons act as a source or a sink for adenosine. By using hENT1 Tg mice, Parkinson *et al.* (2009) found a two-to-threefold increase in [^{3}H]adenosine uptake into synaptosomes. Using hippocampal slice electrophysiology, reduced basal A_1R activity, indicative of reduced adenosine levels, was observed with slices from Tg hENT1 mice, relative to wild type (Wt) littermates. In slices exposed to conditions of hypoxia or OGD, adenosine A_1R activity and thus, adenosine levels, increased in both Wt and Tg slices. However, these changes in adenosine were significantly reduced in slices from Tg hENT1 mice, relative to Wt, indicating that adenosine influx exceeds efflux at neuronal cell membranes, even during ischemic conditions (Chu *et al.,* 2014; Zhang *et al.,* 2011). In behavioral assays, Tg mice also showed a greater response to ethanol and a reduced response to caffeine than Wt littermates; however, no significant differences between heterozygous and homozygous Tg mice were detected (Kost *et al.,* 2011). All these data indicate the source of adenosine in hypoxia or OGD could be derived from

astrocytes directly or by breakdown of extracellular ATP released from astrocytes or another cell type (Parkinson *et al.,* 2009).

The data to date indicate that hENT1 expression leads to increased cellular uptake of adenosine and reduced AR activation. As current studies are from *in vitro* experiments, in this thesis we will extend these data to include *in vivo* studies. As supported by our previous experiments, we hypothesize that faster transport of adenosine from outside to inside by over-expressed hENT1 will decrease extracellular adenosine concentrations and deprive ARs, especially A_1Rs, from adenosine leading to enhanced neuronal injury in a stroke model.

5. Central nervous system effects of adenosine in hypoxia and ischemia

5.1 Physiological roles of adenosine in the CNS

Since it is not exocytotically released, adenosine behaves as an extracellular signaling molecule that influences synaptic transmission without itself being a neurotransmitter. Using G-protein-coupled mechanisms, that not only lead to changes in second-messenger levels but also to the modulation of ion channels (such as Ca^{2+} and K^+ channels), adenosine modulates neuronal activity pre-synaptically by inhibiting or facilitating transmitter release, post-synaptically by affecting the actions of other neurotransmitters, and non-synaptically by hyperpolarizing or depolarizing neurons (Sebastiao and Ribeiro, 2009).

In general, acute (single dose) administration of A_1R agonists have neuroprotective effects, while chronic (multiple dose) treatment can exacerbate neuronal injury (Jacobson et al., 1996). This has been termed “effect inversion” and is likely due to regulation of receptor number and signalling. The degree of effect inversion may depend on drug administration method, frequency, dose and washout time (Jacobson et al., 1996). In traumatic brain injury in mice, 3

weeks of caffeine pre-treatment attenuated lesion size, whereas a single dose 30 min before the same event produced no benefit (Li et al., 2008).

While there is a lot of interest in the role that adenosine plays in conditions such as neurodegenerative disorders, seizures disorders, sleep/wake cycles, pain transmission, blood-brain barrier permeability and substance use disorders, the roles of adenosine and ARs in cerebral blood flow regulation, ischemic brain injury, respiration, apnea of prematurity (AOP) and the pharmacological effects of caffeine are of particular relevance for the research described in this thesis.

5.1.1 Cerebral blood flow (CBF) regulation

The brain has the capacity to modulate cerebral blood flow (CBF) during changes in blood pressure. This phenomenon is known as autoregulation (Kusano *et al.,* 2010). Within 24 hours (h) following middle cerebral artery occlusion, activated astrocytes increase in size and number. Also, stimulation of eN expression by glial cells increases adenosine production in the extracellular environment. This contributes to ischemic preconditioning, which decreases infarct size (Braun *et al.*, 1997). In this situation, adenosine acts as a potent dilator of cerebral vessels and has been implicated in the regulation of CBF (Ngai *et al.,* 2001). Adenosine has high affinity, $A_{2A}R$, and low affinity, $A_{2B}R$, in cerebral resistance vessels. Both receptors are distributed unevenly in CNS, but irrespective of localization, vascular A_2Rs promote vasodilation (Pelligrino *et al.,* 2010, Kusano *et al.,* 2010). In a rat study, intracerebral arterioles responded to $A_{2A}R$ agonists and the effect was greater when $A_{2A}R$ and $A_{2B}R$ agonists were used together. However, there was no effect of the A_1R selective antagonist DPCPX or the A_3R selective antagonist MRS-1191 on adenosine-induced dilation of cerebral arterioles (Ngai *et al.,*

2001). Therefore, the current evidence supports a vasodilator role of A_2Rs but not a vasoconstrictor role of A_1Rs in this process. Vasodilation is very important for the neuroprotection in some neurological events since vasospasm has long been considered the most dreaded acute complication in patients who experience neurologic sequele (Janjua and Mayer, 2003). Although, A_1R role is less evident than A_2R in cerebral vasodynamics, local vasodilator mediators such as nitric oxide (NO) are also involved in adenosine-induced vasodilation (Lin *et al.*, 2006) and A_1R agonists can prevent vasospasm by an NO-preserving effect of A_1Rs on vessels (Lin *et al.*, 2006).

5.2 Ischemic brain injury

Ischemic stroke leads to long-lasting disabilities and it is the second leading cause of death in developed countries. It is also expensive not only in terms of the acute treatment but also the subsequent rehabilitation period (Melani *et al.*, 2014; Markus, 2003). Ischemic stroke commonly accounts for approximately 80% of all stroke cases, and is caused by occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of CBF, OGD and subsequent tissue damage in the affected region. Stroke can affect all ages including newborns at a rate of about 1 in 4000 full term infants (Chang *et al.*, 2005). During stroke, a rapid depletion of ATP, failure of ion pumps with collapse of ion homeostasis, release of excitotoxic neurotransmitters, and calcium overload triggers a series of highly destructive enzymatic cascades that result in apoptosis and necrosis (von Lubitz, 2001). Unfortunately, with the exception of tissue plasminogen activator, which is a time-dependent medication, there are as yet no clear evidence-based strategies for treatment (Chang *et al.*, 2005). Not only adenosine, but also other mechanisms such as acid sensing ion channels have been considered in the etiology,

but current efforts showed no benefit in many studies (O'Bryant *et al.,* 2014). It is well studied that increases in extracellular adenosine levels are neuroprotective during stroke/ischemia (Melani *et al.,* 2014). By activation of A_1Rs Ca^{2+} influx decreases and this results in a reduction in the release of excitatory neurotransmitters as well as increased K^+ and Cl^- ions within a few hours (Borea *et al.,* 2016). In the opposite way, A_{2A}R deficiency or inactivation is considered neuroprotective by decreasing cerebral edema, glutamate outflow and injury size during ischemia and reperfusion in mice (Gui *et al.,* 2009). Surprisingly, recent data suggested a beneficial role of A_{2A}R through attenuation of neuro-inflammation and stimulation of neurogenesis through neurotrophic factors after ischemia (Borea *et al.,* 2016). This might indicate a dual role of A_{2A}R in cerebral ischemia. Results about the role of A_3R in stroke are not fully clear. However, current speculation is that treatment with A_3R agonists protects neurons and glial cells after ischemia by reducing glutamate mediated excitotoxicity (Pedata *et al.,* 2016).

During pathophysiological events such as stroke or brain trauma, adenosine levels can increase up to 100-fold (von Lubitz, 2001; Zamzow *et al.,* 2008b). Adenosine is mainly accepted as neuroprotective in stroke and hypoxia since it inhibits release of excitatory amino acids and inhibits adenylyl cyclase (AC), opens K^+ channels and reduces Ca^{2+} influx into the cell. Adenosine has been shown to modulate the release of glutamate from synaptosomes and to suppress ischemia evoked excitatory amino acid release in hippocampus, striatum and cortex. Adenosine blocks Ca^{2+} spikes in hippocampal slices and shows negative feedback on glutamate release (Kitagawa *et al.,* 2002; Williams-Karnesky and Stenzel-Poore, 2009; Thauerer *et al.,* 2012). This neuroprotection has been largely attributed to activation of A_1R (Li *et al.,* 2008). In contrast, A_{2A}R promote release of glutamate under both normoxic and ischemic conditions

(Melani *et al.,* 2009). The response to adenosine is mainly a balance between A_1R and $A_{2A}R$ activations (Okada *et al.,* 2001; Cunha, 2005).

Adenosine suppresses various cytotoxic processes, such as cytokine-induced apoptosis. In the brain, both neuronal and glial cell functions are regulated by adenosine. By giving an $A_{2A}R$ agonist, ATL146e, Cassada *et al.* (2002) found a reduced apoptosis and marked improvement after spinal cord trauma in rabbits. Similarly, adenosine analogues and endogenously produced adenosine acting on A_1R and A_3R showed a protection on primary mouse astrocytes from the hypoxic insult (Bjorklund *et al.,* 2008). A_1R stimulation activates protein phosphatases and upregulates glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit A1 and downregulates AMPA receptor subunit A2, in cerebral ischemia (Stockwell *et al.,* 2016). In contrast, others report $A_{2A}R$ blockade is antiapoptotic. Silva *et al.* (2007) induced apoptosis of rat hippocampal neurons by exposure to staurosporine and the process was blocked by $A_{2A}R$ antagonists, SCH58261 and ZM241385. Their suggested mechanism was synaptic mitochondria dysfunction and caspase-3 activation in synapses. Other possible mechanisms are activation of mitogen-activated protein kinase (MAPK) (Schulte and Fredholm, 2003) and phospho-extracellular signal-regulated kinase 1/2 pathways and inhibition of diacylglycerol signaling with consequent reduction of inflammatory and excitotoxic cascades (Mohamed *et al.,* 2016). Additional mechanisms, such as reduced hippocampal microglial activation, glial tumor necrosis factor-alpha (TNF-α) and brain-derived neurotrophic factor expression, glutamate, inducible nitric oxide synthase and thiobarbituric acid reactive substances related to $A_{2A}Rs$ have also been proposed (Mohamed *et al.,* 2016). All this evidence suggests a protective or detrimental role of adenosine receptors on apoptosis based on the receptor subtype.

6. Experimental stroke models

In experimental stroke models, mice and rats are the most commonly used laboratory animals but various other animals like rabbits, gerbils, cats, dogs, pigs and monkeys have been used with various advantages and disadvantages owing to which their selection for a particular investigation is made (Kumar *et al.,* 2016). As was well reviewed by Durukan and Tatlisumak (2007) there are several techniques that have been used as stroke models. For studies requiring focal cerebral ischemia, surgical occlusion of middle cerebral artery (MCA) or other vessels require craniectomy (Kumar *et al.,* 2016). A reversible intraluminal MCA occlusion by using a monofilament has also been widely used; a model that requires microsurgical skill (Kumar *et al.,* 2016; Braeuninger and Kleinschnitz, 2009; Shahjouei *et al.,* 2016). Other methods that do not require cariotomy include embolic models, photothrombosis, and ET-1 injection.

6.1 Endothelin-1 overview and its use in stroke model

ET-1 is an endothelium derived 21 amino acid peptide. It is a very potent and long-acting vasoconstrictor (Fuxe *et al.,* 1989; Reid *et al.,* 1995). It is localized in and released by endothelial cells in both the peripheral and cerebral vasculature and is also localized within neurons in certain brain areas such as the nucleus of the solitary tract, area of rostroventrolateral medulla, and hypothalamus in which it contributes to central regulation of blood pressure (Reid *et al.,* 1995). The ET family contains three members, namely, ET-1 (expressed in endothelial cells), ET-2 (expressed in kidneys and jejunum), and ET-3 (expressed in adrenal glands, jejunum, and kidneys). Vascular endothelial cells secrete both ET-1 and nitric oxide and both are in a dynamic balance to regulate vascular tone (Yu *et al.,* 2015). Thus, ET-1 could serve not only

a role in local control of cerebral vascular tone but may also have central neurotransmitter and neuromodulator functions (Reid *et al.,* 1995).

Local application of ET-1 to a brain supplying artery or on the brain surface (Braeuninger and Kleinschnitz, 2009) has been used for decades to produce an experimental stroke in rats. Fuxe *et al.* (1989) reported that intrastriatal (IS) injection of ET-1 is effective in male rats. They found that 1 microgram (µg) ET-1 is much more effective than smaller doses. Similar results were reported in additional studies with rats (Gilmour *et al.,* 2004; Windle *et al.,* 2006). Due to the advantages of using genetically altered mice in stroke experiments, Horie *et al.,* (2008) tested and compared the efficacy of ET-1 on rats and different mouse strains. IC and IS injections of 1 to 5 µg ET-1 in single or multiple injections did not cause stroke injury in mice but worked well in rats. However, no CBF measurement was done; therefore no information was provided about vasoconstriction response to ET-1. Based on their results, ET-1 was considered unsuccessful for mouse stroke models in some reviews (Bacigaluppi *et al.,* 2010). As described in following chapters, we have used ET-1 in mice to establish cortex specific stroke and it was found to be relatively simple and effective, yet safer than other methods (Figure 2).

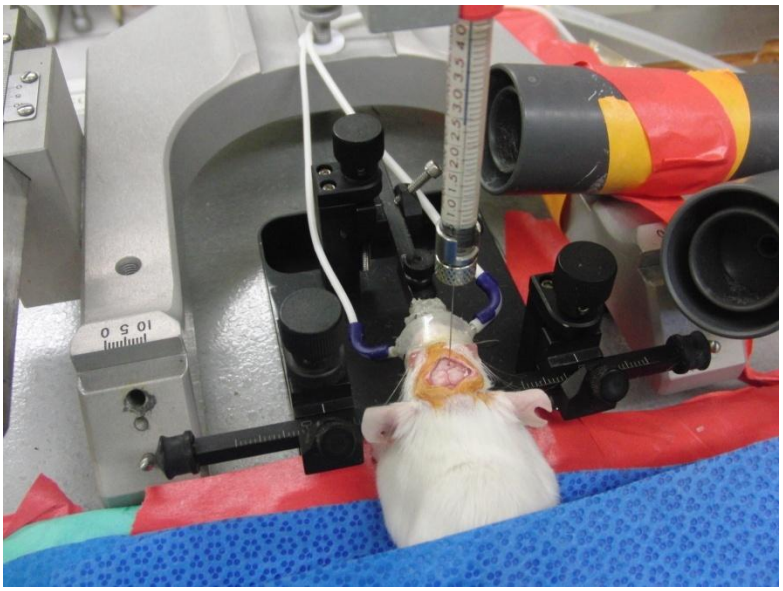


Figure 2: ET-1 injection in mouse model. Under isoflurane anesthesia, mouse is placed on sterotaxic apparatus, and head is stabilized. After scalp cleaning and local anesthesia, scalp is opened. A syringe containing drug is used to infuse the appropriate drug dose into desired coordinates. [Picture was taken by HS.]

6.2 Magnetic resonance imaging as a diagnostic tool for stroke

The extent of brain damage following an injury is determined not only by the primary insult itself, but also by the subsequent neuronal loss due to secondary brain injury. However, it is a multifactorial process and includes cerebral hypoperfusion, arterial hypotension, hypoxemia and flow–metabolism uncoupling, as well as the metabolic, immunological, and biochemical changes (local inflammatory changes, Ca^{2+} and excitatory amino acid imbalances, excitotoxic injury, and apoptosis) initiated by the original insult (Dagal and Lam, 2011). CBF affects and is

affected by these problems. It has also been reported that a decrease in CBF to 20% of control levels is required for stroke injury (Siesjo, 1992). There are many ways to measure CBF and perfusion. Position emission tomography, perfusion weighted MRI, stable xenon tomography, single photon emission computed tomography, computed tomography perfusion scan, thermal diffusion flowmetry, laser Doppler flowmetry, transcranial Doppler ultrasonography, jugular venous oximetry, near-infrared spectroscopy and intracerebral microdialysis are used for this purpose in human and animals (Dagal and Lam, 2011).

MRI plays an increasingly important role in studying mechanisms of brain injury (Hoehn *et al.,* 2001). The advantage of using an MRI-based perfusion approach is its non-invasiveness, possibility of time-course assessment of tissue perfusion, ability to discern morphological features, metabolism, and function (Hoehn *et al.,* 2001; Detre *et al.,* 2009). The combination of T2- and/or diffusion-weighted MRI with perfusion-weighted MRI allows concurrent assessment of tissue lesions and perfusion alterations. Two perfusion weighted MRI strategies that are frequently applied are dynamic susceptibility contrast-enhanced and arterial spin labeling MRI (Dijkhuizen, 2011; Shen and Duong, 2016). Dynamic susceptibility contrast-enhanced MRI allows calculation of various hemodynamic parameters. However, arterial spin labeling MRI is based on spatially selective inversion or saturation of incoming arterial spins and subsequent detection of labelled blood water in perfused tissue, which enables quantification of cerebral blood flow. Both methods have been applied in rodent models of traumatic brain injury, and have provided insights into the development of focal as well as global post-traumatic cerebral hypoperfusion (Dijkhuizen, 2011).

The application of MRI to studies of the brain is widely accepted and used in many experimental studies today because of its fantastic battery of measurement sequences leading to

information about structural, hemodynamic, metabolic, and even functional aspects of the brain (Kallur and Hoehn, 2011). Necrosis develops from 6 hours up to 4 days after ischemia and is further aggravated by inflammatory processes. After 1 to 2 weeks, a loose connective tissue matrix is formed along the infarct borders. The necrotic cells are completely resorbed approximately 2 weeks later, with macrophages remaining in the tissue adjacent to the infarct area (Wegener *et al.,* 2006). Although diffusion weighted MRI is generally accepted as a superior method to T2-weighted MRI, both methods have been shown to be predictive for final infarct volume when they were performed at 24 hour after hypoxic insult (Wang *et al.,* 2007). Moreover, optimal timing of T2-weighted MRI in rodents is a subject of many studies. Peeling *et al*. (2001) occluded MCA and generated a stroke in rats. They found a highly significant correlation in infarct size between T2-weighted brain MRI performed on 2 and 7 days and histopathologic infarct size in the cortex, subcortex in brain samples taken on 30 and 60 days following the occlusion. Albensi *et al.* (1998) also showed that hyperintensities on T2-weighted MRI were evident as early as 1 to 6 hours and peaked between 24 and 48 hours after hypoxia on P7 rat pups. Areas of hyperintensity identified at 72 hours after hypoxia corresponded well to permanently damaged (infarct) brain parenchyma as demonstrated by post-mortem histopathology.

7. Respiration and adenosine receptors

Several studies have focused on the relationship between adenosine and breathing. Koos *et al*. (2001) showed that A_1Rs are not significant modulators of respiratory rhythm, but they have tonic depressant effect on BS breathing center, and therefore reduce the amplitude of breathing. In contrast, A_{2A}R have a modulating effect on respiration rate and reduce the

incidence of breathing. Thus, it appears that both A_1 and A_{2A}Rs have a central effect on breathing.

However, some other studies show the effect of adenosine on respiratory drive by effects on carotid body, a peripheral chemosensor of the body. The carotid body mainly contains A_{2A} and A_{2B}Rs. Interestingly, adenosine has a stimulatory effect on respiratory work driven by carotid body through A_{2A}Rs by increasing respiratory rate 31% (Conde *et al.,* 2009). In human studies, adenosine injection can cause hyperventilation, chest pain and dyspnea (Watt *et al.,* 1987). From these studies, it seems that adenosine acts differently on breathing through its central and peripheral receptors.

Endogenous extracellular adenosine has a suppressive effect on fetal and neonatal breathing (Zaidi *et al.,* 2006). A prominent role for adenosine in neonatal respiratory inhibition is suggested by the ability of the nonspecific AR antagonists, theophylline and caffeine to decrease the incidence of AOP (Martin *et al.,* 2004). Especially A_1Rs in the brainstem have been identified in many of the areas that are related to the respiratory network, such as ventral respiratory group and the pontine respiratory area, medial parabrachial and Kolliker-Fuse nuclei (Gaytan *et al.,* 2006). Centrally applied adenosine analogs depress respiratory rate and depth via A_1Rs in the BS (Herlenius *et al.,* 2002). In another study, by using A_1R knockout newborn mice, it was shown that A_1Rs are critical components for stabilization of respiration (Heitzmann *et al.,* 2016). However, Zaidi *et al.* (2006) stated the importance of γ-amino butyric acid (GABA) related transmission and suggested that adenosine and A_{2A}Rs expressed by medullary GABA containing cells play an important role in adenosine-induced respiratory depression. Indeed, A_{2A}R agonist injection stimulated respiratory inhibition and it was reversed by bicuculline, a selective $GABA_A$ antagonist (Martin *et al.,* 2004).

7.1 Beneficial and adverse effects of caffeine, a neonatal respiratory stimulant and an AR antagonist

Caffeine is the most widely consumed behaviorally active substance in the world. Caffeine ingestion is well known to give a dose dependent increase in energetic arousal and to help concentration (Nehlig, 2016). As a very active medical substance, caffeine plays a role against or contributes to Alzheimer`s disease, Parkinson`s disease, depression, anxiety, cognitive enhancement, neuroprotection, diuresis, vomiting, apnea and chronic obstructive pulmonary disease (Daly, 2007; Fredholm *et al.,* 1999; Nehlig, 2016; Mohan *et al.,* 2015).

Most of caffeine is consumed through beverages/foods and caffeine content of these various beverages/foods ranges from 40 to 180 mg/150 milliliter (ml) for coffee to 24 to 50 mg/150 ml for tea, 15 to 29 mg/180 ml for cola, 2 to 7 mg/150 ml for cocoa, and 1 to 36 mg/28 gram (g) for chocolate (Fredholm *et al.,* 1999). Caffeine, the major behavioral stimulant present in coffee, was isolated in 1820 and the correct structure of this methylxanthine was finally established in the last decade of that century (Daly, 2007). In animals and man, it is absorbed rapidly and completely from gastrointestinal tract. In man, 99% of the administered dose was absorbed in 45 minutes (min), mainly from the small intestine but also 20% from the stomach (Fredholm *et al.,* 1999; Arnaud, 2011). The absolute bioavailability of caffeine (5 milligram (mg)/ kg) in healthy adult male volunteers shows a rapid oral absorption with the time to reach the peak plasma concentration of 29.8 ± 8.1 min and a peak plasma concentration of 10.0 ± 1.0 μg/ml (Arnaud, 2011). Caffeine metabolism is faster in men than women, also genetic factors affect metabolism rate (Rasmussen *et al.,* 2002). Caffeine is widely distributed in the body with tissue levels approximately proportional to their water content (Fredholm *et al.,* 1999). Serum protein binding by caffeine is low. CYP1A2, one of the most important drug-metabolizing

enzymes accounting for nearly 15% of the cytochrome P450 in the human liver (Rasmussen *et al.,* 2002), metabolizes caffeine into three dimethylxanthines: paraxanthine, which increases lipolysis, leading to elevated glycerol and free fatty acid levels in the blood plasma; theobromine, which dilates blood vessels and increases urine volume; and theophylline, which relaxes smooth muscles of the bronchi, and is used to treat asthma (Ribeiro and Sebastiao, 2010). However, because the metabolism of caffeine differs between rodents and humans and the half-life of the methylxanthine is much shorter in rats (0.7–1.2 h) than in humans (2.5– 4.5 h), it seems reasonable to correct for the metabolic body weight when comparing animal and human doses. Thus, it is generally assumed that 10 mg/kg in a rat represents about 250 mg of caffeine in a human weighing 70 kg (3.5mg/kg), and that this would correspond to about 2 to 3 cups of coffee (Leon *et al.,* 2002). However, average caffeine intake varies from 250 to 600 mg/day, which represent 3 to 7 cups/day of coffee consumption (Fredholm *et al.,* 1999; Leon *et al.*, 2002).

7.1.1 Mechanism of effect

Caffeine affects diverse systems and therefore its biological effects extend to many pathological and physiological endpoints. Its primary mechanism of action is as an antagonist on ARs (Ribeiro and Sebastiao, 2010). The brain concentration of caffeine is about 80% to 100% of its plasma concentration (Constenla *et al.,* 2010; Nehlig, 2016). Caffeine's effect on CNS changes with chronic consumption and these changes are affected by drug administration method, frequency, dose, and washout time before measuring functional changes (Constenla *et al.,* 2010; Daly, 2007). At the low micromolar range caffeine antagonizes ARs, mainly A_1R, $A_{2A}R$ and some degree of A_3Rs (Fredholm *et al.,* 1999). Caffeine activates the release of mainly excitatory transmitters; these are more strongly inhibited by adenosine than inhibitory

neurotransmitters (Nehlig, 2016). In preterm infants, A_1R activation may contribute to white matter injury in the preterm infant by altering oligodendrocyte development. In models of perinatal brain injury, caffeine is neuroprotective against periventricular white matter injury and hypoxic-ischemic encephalopathy (Rivkees and Wendler, 2011). When rats access caffeine in their drinking water (1 g/liter (l)) for a period of 4 weeks, rats consume an average amount of 73 ± 3 mg/kg/day with plasma concentration of caffeine of 32 ± 4 micromolar (μM), which is the recommended dose to probe the effects of caffeine on synaptic plasticity in hippocampal slices and is equivalent of 6 cups of daily coffee consumption in humans (Constenla *et al.,* 2010). In higher doses caffeine inhibits phosphodiesterases, also it releases Ca^{2+} from intracellular stores, and it antagonizes $GABA_A$ receptors (Chen *et al.,* 2010; Daly, 2007). Caffeine significantly blocks $A_{2A}R$ (most potent), and A_1Rs at the low concentrations achieved after a single cup of coffee. To inhibit phosphodiesterase, 20 times higher concentrations are required; to block $GABA_A$ receptors, 40 times higher concentrations; and to mobilize intracellular Ca^{2+} depots, concentrations of 100 times higher are needed. There are many other biological targets for caffeine but they require very high concentrations (Fredholm *et al.*, 1999).

7.1.2 Adverse effects

In rats the median lethal dose of caffeine is around 200 mg/kg (Fredholm *et al.*, 1999). Adverse effects of caffeine are related to the dose and include anxiety, hypertension, drug interactions, and withdrawal symptoms (Daly, 2007). Overuse and dependency occurs after consumption of caffeine in large amounts, and in particular over extended periods of time, inducing caffeinism which is a combination of caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety,

tremulousness, muscle twitching, hyperreflexia, insomnia, headaches and respiratory alkalosis (Nehlig, 2016). Caffeine increases production of stomach acid; high usage over time can lead to peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease (Ribero and Sebastiao, 2010). Tolerance against caffeine is relatively fast after heavy doses such as 400 mg of caffeine 3 times a day for 7 days or 300 mg 3 times per day for 18 days (Fredholm *et al.,* 1999; Ribero and Sebastiao, 2010). In a rat study, caffeine was administered for 14 days at 1 g/l concentration in drinking water and motor activation effects of caffeine were diminished because of tolerance to mostly A_1R blockage (Karcz-Kubicha *et al.,* 2003). Caffeine causes a variety of withdrawal symptoms which include weariness, apathy, weakness, drowsiness, and decreased motor behavior (Fredholm *et al.,* 1999). Caffeine withdrawal induces a 2-fold decrease in rat locomotor activity. This effect lasts for about 4 days and is dose-dependent and maximal on the second day. In humans, withdrawal usually lasts up to 7 days (Fredholm *et al.,* 1999). However, individual genetic differences could affect responses to caffeine and it may occur at the metabolic (pharmacokinetic) or at the drug-receptor level (pharmacodynamic), and they can contribute to the quality and magnitude of direct drug effects as well as to consumption of the drug (Yang *et al.,* 2010).

7.1.3 Pregnancy and caffeine

Caffeine is one of the most widely used neuroactive substances being consumed by 90% of pregnant women with an estimated average consumption in the range of 200-300 mg/day (or equivalent to 2-3 cups of brewed coffee) (Yang *et al.,* 2010). There is no BBB to caffeine in the adult or the fetal animal (Fredholm *et al.,* 1999). In gestation day- 20 pregnant rats, a single dose of caffeine at 5 or 25 mg/kg showed similar values in fetal and adult plasma. The concentration

of caffeine in the fetal brain was in equilibrium with the fetal circulation. However, theophylline, paraxanthine and theobromine accumulated in the fetal brain to 3-times the circulating levels (Wilkinson and Pollard, 1993). When caffeine was given at 25 mg/kg or 5 mg/kg to gestation day 20 pregnant rat, the elimination phase was prolonged and there was a significant difference between half-life for the pregnant (8.9 h) vs. non pregnant rat (3.8 h) at 25 mg/kg but no difference at 5 mg/kg dose (Abdi *et al.,* 1993). Caffeine, when administered in moderate doses to rats during pregnancy, has been shown to cause significant fetal and postnatal growth retardation resulting in smaller adults (Pollard *et al.,* 1987). Malformations have been demonstrated in mice at 50-75 mg/kg of caffeine, whereas the lowest dose usually needed to induce malformations is 80 mg/kg in rats. However, when caffeine is administered in fractioned amounts during the day, 330 mg/kg/day was necessary to reach teratogenicity such as limb or craniofacial malformations (Nehlig and Debry, 1994). Caffeine was shown to be teratogenic in several species, including rabbits, mice and rats in high levels intake, but no human data confirmed this finding (Leon *et al.,* 2002). An epidemiological meta analysis has not been able to detect significant risks for teratogenic effects of caffeine exposure in humans (Browne, 2006). Some other studies have raised concerns that maternal consumption of caffeine in humans (more than 300 mg/day) can decrease fertility and increase spontaneous abortions (Aden, 2011). In a large scale study, caffeine consumption throughout pregnancy was associated with an increased risk of fetal growth restriction: odds ratio (OR) 1.2 (95% confidence interval (CI) of 0.9 to 1.6) for 100-199 mg/day, OR 1.5 (1.1 to 2.1) for 200-299 mg/day and, OR 1.4 (1.0 to 2.0) for >300mg/day, compared with <100 mg/day. These results were explained by decreasing intervillous blood flow in the placenta after caffeine intake (CARE Study group, 2008). In another large study of 18,478 singleton pregnancies, a significant association between coffee and still birth was revealed for

women consuming 8 or more cups of coffee per day during pregnancy. Adjustment for smoking and alcohol intake during pregnancy did not significantly affect the results (Wisborg *et al.,* 2003). However, there is not enough data about association between caffeine and prematurity and also there is no strong evidence for advising mothers to avoid caffeine during the last two thirds of pregnancy (Aden, 2011). Interestingly, there are some human data which show that high caffeine intake during pregnancy results in withdrawal symptoms which usually appear as irritability, jitteriness and vomiting in their newborn babies (Martin *et al.,* 2007b; McGowan *et al.,* 1988).

7.1.4 Newborn and caffeine

Caffeine and its metabolites pass freely through the placenta to the fetus and to the milk during lactation (Abdi *et al.,* 1993). The plasma half-life of caffeine is very much longer in human neonates than in adults and ranges from 40 to 230 h (mean of 100 h) in neonates compared to 4 hours in adults, reflecting the inability of the neonatal liver to metabolise the drug via CYP1A2. The half-life of caffeine decreases with advancement of age as its metabolism develops, and it reaches an adult level at the age of 5–6 months (Aranda *et al.,* 1979). Elimination in newborn is also reported to be 2.5 to 16.8 ml/kg/h (mean; 8 9 ml/kg/h) (Aranda *et al.,* 1979).

Alteration of astrocytogenesis via $A_{2A}R$ blockade by caffeine during brain development has been reported as detrimental for long-term brain functions in mice pups (Desfrere *et al.,* 2007). However, there is no clear evidence about such a detrimental effect of caffeine in human studies. But, in human newborns treated with methylxanthines for apnea, a decrease in CBF up to 22% has been reported at 2 hours of the administration of caffeine and it can be prevented if

methylxanthine induced hypocapnea is corrected (Fredholm *et al.*, 1999; Hoecker *et al.*, 2002). However, these changes are moderate and the decrease in CBF could be compensated for by an increase in oxygen and glucose extraction, because the consumption of moderate amounts of caffeine has positive effects on alertness (Fredholm *et al.*, 1999). Caffeine administration on P2 to P6 resulted in hyperalgesia in hot plate test, less anxiety than controls in the elevated plus-maze and dark–light transition, and impairment in step-through avoidance learning test on P35 to P42. Moreover, the responses to A_1R and A_2R agonists were enhanced (Pan and Chen, 2007). Indeed, chronic therapy with receptor-specific antagonists in animals and man has been reported to induce an enhanced response to agonists following removal of the antagonists which indicates a withdrawal syndrome (Green and Stiles, 1986). In current practise, caffeine is the most common medication in preterm babies and the maintenance dose is 2.5 mg/kg/day caffeine base (5 mg/kg/day caffeine citrate) and this was accepted as enough to maintain therapeutic plasma caffeine levels in 94.8% of preterm infants (Natarajan *et al.*, 2007). A recent international trial has impacted our understanding of caffeine treatment of neonates. Among 2006 preterm newborn infants, treatment with caffeine as compared with placebo reduced the incidence of cerebral palsy (4.4% vs.7.3%; adjusted OR, 0.58; 95% CI, 0.39 to 0.87; p = 0.009) and of cognitive delay (33.8% vs. 38.3%; adjusted OR, 0.81; 95% CI, 0.66 to 0.99; p = 0.04). The rates of death, deafness, and blindness and the mean percentiles for height, weight, and head circumference at follow-up did not differ significantly between the two groups (Schmidt *et al.*, 2007).

7.1.5 Caffeine, ARs and apnea

It is known for long time that theophylline acts as an AR antagonist and reverses the depressive effect of L-N^6-(phenylisopropyl) adenosine, a long-acting analogue of adenosine (Eldridge *et al.,* 1985). Methylxanthines, including caffeine, exert their effects by competitive inhibition of brainstem A_1R and $A_{2A}R$ located on groups of neurons involved in the generation of respiratory rhythm (Comer *et al.,* 2001). Respiratory rhythm generation in the medulla and its inhibition by the pons were found to be more pronounced in fetuses and pups of rats treated with caffeine (Herlenius *et al.,* 2002). By giving 20 mg/kg doses of caffeine Yoder *et al.* (2005) have reported better lung function and decreased CO_2, and increased compliance in preterm baboons. On the other hand, Gaytan *et al.* (2006) treated newborn rat pups with caffeine for 1 week and they found a significant increase in A_1Rs in regions of the brain associated with respiratory control at P6 with caffeine treatment (Gaytan *et al.,* 2006), and such changes were associated with increased minute ventilation in response to hypercapnia (Montandon *et al.,* 2006; Montandon *et al.,* 2007). Chronic caffeine consumption (0.2 g/l) of pregnant rats resulted in respiratory disturbance (tachypne and apnea) in newborn pups. Caffeine through mothers' milk during nursing period corrected this problem (Bodineau *et al.*, 2006). Changes in respiratory control in caffeine treated rats at P3 to P12 period have persisted into 3–4 months old adulthood period (Montandon *et al.,* 2006). Similarly, Julien (2011) found an alteration to hypoxic response in newborn rats after 10 days of treatment of caffeine but not domperidone, a peripheral dopamine D2 and D3 receptor antagonist. Methylxanthines also stimulate lung maturation. In fetal rabbit lung, theophylline improved surface tension and stimulated surfactant synthesis (Corbet *et al.,* 1978).

8. Summary

Current literature has provided many roles for adenosine and its receptors in almost all organs and systems. This is not surprising since adenosine is a metabolite of ATP and any condition such as reduced oxygen and glucose availability and/or increased demand to cellular energy results in increased intracellular levels of adenosine by ATP breakdown (Chu *et al.,* 2014). In addition, some other studies showed extracellular modulation of adenosine by astrocytes (Martin *et al*, 2007a; Zamzow *et al.,* 2008b). The formation of adenosine is strictly dependent on the metabolic state of a cell and its physiological actions have been considered as "ubiqituous hemostatic substance and signal of life" by many authors since adenosine is "omnipresent" i.e. exists in all cells (Ribeiro *et al.,* 2002).

AR have different affinity for adenosine with A_1R and $A_{2A}R$ having the highest affinity followed by A_3R then by $A_{2B}R$ (Ribeiro and Sebastiao, 2010). All cell types in the CNS possess one or more of these receptors, with neuronal receptors existing on both presynaptic terminals and postsynaptic membranes (Stone *et al.,* 2009). These receptors are very functional and any polymorphism might result in serious health conditions, such as febrile status epilepticus and psychomotor vigilance (Bodenmann *et al.,* 2012; Shinohara *et al.,* 2013). In addition, ARs by themselves contribute to or prevent many physiopathological conditions such as sleep-wake, cognition, learning, memory, epilepsy, depression, ischemia, pain, inflammation, autoimmune diseases, inflammatory bone loss, hepatic fibrosis, wound healing and remodelling, lung injury, intestinal inflammation, cancer, stroke, etc. (Borea *et al.,* 2016; Ribeiro and Sebastiao, 2010; Huang *et al.,* 2014). Therefore, modification of adenosine release or receptors by agonists and antagonists may affect the course of above mentioned conditions (Stone *et al.,* 2009). In the CNS adenosine acts as an endogenous modulator on brain functions, fine-tuning glial function,

blood flow, inhibitory and excitatory synaptic transmission, and affecting the outcome of brain injuries, stroke, ischemia and epileptic seizures (Di Angelantonio *et al.,* 2015; Pedata *et al.,* 2015).

Modification of local ATP and adenosine levels by inhibition of plasma membrane adenosine transporters/ATP hemi-channels are also studied (Pedata *et al.,* 2015). Indeed, ENT1 and ENT2 are important mediators of adenosine influx or efflux related to intracellular or extracellular adenosine concentrations (Parkinson *et al.,* 2005; Xu *et al.,* 2015). ENT1 expression could affect dynamic changes in endogenous adenosine levels and alter responses to the drugs (Parkinson *et al.,* 2009). Therefore, it could be a potential target for detrimental or protective features of adenosine. Another important way to get benefit from adenosine`s neuroprotection is through modification of its receptor activity. Although it is a non-selective AR antagonist, caffeine is shown as a neuroprotective agent against neurodegenerative diseases in elderly people and as a neurocognitive healer in extreme premature babies (Cunha, 2005; Rivera-Oliver and Diaz-Rios, 2014; Chen *et al.,* 2010; Schmidt *et al.,* 2007). Also, caffeine is the most widely consumed substance through caffeinated beverages over the world. It is not clear and still is questionable if caffeine consumption, especially in higher dose, has any ontogenic effect on ARs. It is also not clear, if any modification in ARs by caffeine consumption during the pregnancy might change the ventilatory response of newborns. Therefore, we established an *in vivo* and *in vitro* model and focused on two major problems in two extreme ages; stroke a major problem of elderly people and apnea a major problem of early newborn people. The shared point of these two diverse conditions was adenosine and its receptors.

9. Research Objectives

Two main lines of research were addressed in this thesis. First, we used hENT1 transgenic mouse model established by Dr. Parkinson (Parkinson *et al*., 2009). As described above, it is well known that adenosine is produced in large quantities in response to hypoxic/ischemic conditions. In brain, this adenosine provides a level of neuroprotection by activating A_1Rs, reducing glutamate neurotransmission, reducing Ca^{2+} influx into neurons and promoting K^+ efflux from neurons. However, there is an ongoing debate about whether the source of this adenosine is mostly intra- or extracellular. Based on our previous *in vitro* experience, we suggest that the source of adenosine is mostly in the extracellular environment subsequent to ATP release from astrocytes. If this is the case, then the direction of adenosine transport should be toward inside cells because of the higher concentration in extracellular environment. If this is true, then ENT1 overactivity will decrease extracellular adenosine faster and it should result in increased neurotoxicity in stroke conditions. Some other experiments supported this theory from opposite way by blocking of ENT1activity using inhibitors; this resulted in slowed adenosine clearance from extracellular area (Nguyen *et al.,* 2015, Cui *et al.,* 2013) and led to increased neuroprotection (Cui *et al.,* 2013). We tested the effect of increased transporter activity using a hENT1 transgenic mice, which expresses hENT1 selectively in neurons (Parkinson *et al*., 2009). It is important to understand this mechanism because it would affect the development of treatment strategies against hypoxia, stroke or other neurodetrimental issues. The first chapters of this thesis will try to answer to this question *in vivo*.

In Chapter II and Chapter III we used a relatively novel *in vivo* stroke model, namely IC injection of ET-1 to produce a long lasting vasospasm and associated ischemic injury in hENT1 transgenic mice and wild type littermates. We proposed that if adenosine is primarily formed

within neurons during an ischemic event, then over-expression of ENT1 would allow enhanced cellular release of adenosine to the extracellular space and would cause stronger A_1R activation and reduced ischemic infarct sizes. However, if the opposite is true and adenosine is primarily produced extracellularly following cellular release and metabolism of ATP by astrocytes, then over-expression of ENT1 would lead to enhanced cellular uptake of adenosine, reduced A_1R activity and increased ischemic infarct sizes. We extended these studies by testing the effects of caffeine, a non-selective adenosine receptor antagonist, and DPCPX, a selective A_1R antagonist.

For the second line of research in this thesis, we examined the effect of prenatal exposure to caffeine on pregnancy outcomes, brain A_1R and $A_{2A}R$ expression and abundance, and respiratory responses to a hypoxic challenge. These studies are described in Chapters IV and V and were undertaken in an attempt to address shortcomings in our knowledge of the mechanism of action and variable responses to caffeine among human infants with AOP. For these infants, caffeine is the preferred clinical treatment, but as caffeine is widely consumed by adults, including pregnant women world wide, fetal exposure at varying levels is common. We proposed that an effect of prenatal caffeine exposure on post-natal AR functions and caffeine responses could lead to reduced effectiveness of caffeine to treat post-natal treatment of hypoxic respiratory responses.

Chapter II:

Intracortical injection of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter[1]

1. Background

Stroke is a leading cause of death and disabilities in developed countries. During stroke, a rapid depletion of ATP, dysregulation of ion channels and pumps, collapse of ion homeostasis, release of excitotoxic neurotransmitters, and intracellular Ca^{2+} overload triggers a series of enzymatic cascades that result in neuronal death (Szydlowska *et al.,* 2010; Deb *et al.,* 2010). Unfortunately, with the exception of tissue plasminogen activator, there are as yet no clear evidence-based strategies for treatment (Deb *et al.,* 2010).

Adenosine is a neuromodulator that acts through a family of metabotropic receptors. Activation of A_1Rs, which are widely distributed in brain, has neuroprotective effects. In contrast, activation of A_{2A}R, which are the most abundant in basal ganglia, can promote cell injury (Gui *et al.,* 2009). Adenosine can be formed extracellularly from catabolism of ATP by a cascade which includes of eN (Matsuoka and Ohkubo, 2004), or can be formed intracellularly from ATP consumed in energy requiring processes followed by hydrolysis of AMP by cN. ENTs mediate cellular influx or efflux of adenosine and other nucleosides as dictated by their concentration gradients (Zamzow *et al.,* 2008a). During pathophysiological events such as stroke or brain trauma, adenosine levels can increase up to 100-fold; the origin of this adenosine has been addressed *in vitro* using pharmacological tools but little information from *in vivo* studies is available (Parkinson *et al.,* 2000; Frenguelli *et al.,* 2007).

[1] A version of this chapter has been published as Soylu, H., D. Zhang, R. Buist, M. Martin, B.C. Albensi and F.E. Parkinson. 2012. Intracortical injection of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter. Exp. Transl. Stroke Med. 4:4.

Genetically altered mice can be used to investigate ischemic injury at the molecular level (Horie *et al.,* 2008). One method for producing a cerebral infarct in rodents is intracerebral injection of the vasoconstrictor peptide ET-1 (Fuxe *et al.,* 1989; Gilmour *et al.,* 2004; Wang *et al.,* 2007). Compared to other methods for producing stroke, such as distal MCA occlusion or thromboembolic models, ET-1 induced focal lesions are relatively simple and reproducible (Bacigaluppi *et al.,* 2010). Moreover, ET-1 is not directly neurotoxic but produces a temporary reduction in blood flow for several hours at the injection site (Windle *et al.,* 2006; Luo and Grammas, 2010).

In the present study ET-1 or saline was administered by IC injection to Wt and Tg mice with neuron-specific expression of hENT1 (Parkinson *et al.,* 2009). Local changes in CBF and development of a cerebral infarct were monitored by MRI to test whether ENT1 over-expression affected stroke injury in a brain region expressing adenosine A_1Rs. The role of ARs in responses of Wt and Tg mice to ET-1 were examined with the antagonist caffeine.

Our aim was to test the hypothesis that over expression of hENT1 increases ischemic damage in an *in vivo* stroke model, extending our findings from our previous *in vitro* study (Zhang *et al.,* 2011). A second aim was to test whether the non-selective AR antagonist, caffeine, makes any different action on ischemic injury in ENT1 over expressed and Wt mice.

2. Methods

2.1 Mice

CD1 mice with neuronal expression of hENT1 were generated as previously described (Parkinson *et al.,* 2009). To compare Tg to Wt mice, heterozygous Tg male mice were bred to Wt females and 8-10 week old (35-40 g) heterozygous Tg and Wt male littermates were used.

All procedures and all outcome analyses were performed by individuals who were blinded to the genotype of the mice. All procedures with animals were approved by the University of Manitoba Animal Protocol Management and Review Committee.

2.2 Surgical procedure

Mice were anesthetized initially with 5% isoflurane for induction then maintained with 1.5-2% isoflurane in 30% oxygen and 70% nitrogen. Rectal temperature was kept between 37-37.5°C through a thermal pad during the procedure and was maintained by a heating lamp during the recovery. Mice were placed in a stereotaxic apparatus. Following a midline incision to the scalp, a small burr hole was placed in the right hemisphere. A 5 microliter (μl) Hamilton syringe was used to inject 1 microgram (μg) ET-1 (Sigma-Aldrich Canada, Oakville, ON, Canada) (400 picomole (pmol) in 1 μl of saline) or saline (1 μl) to the cortex in 5 min. One group of mice received caffeine base (25 mg/kg) by IP injection 30 min prior to IC injection of ET-1. The stereotaxic coordinates were determined from a mouse atlas (Paxinos and Franklin, 2001) and were 1.0 mm anterior, and 1.0 mm lateral, to bregma and 1.2 mm below the pia. The needle was left *in situ* for 5 min to prevent back flow of ET-1 or saline before being slowly withdrawn. The hole was covered by bone wax and the incision was sutured.

2.3 Magnetic resonance imaging

Mice were examined with perfusion-weighted MRI at 4 and 48 hours post-injection to confirm decreased CBF. In addition, they were examined with T2 weighted MRI at 48 hours to determine injury size. A Bruker Biospec 7 T/21 cm spectrometer (Bruker BioSpin, Karlsruhe, Germany) with a quadrature volume coil (National Research Council, Winnipeg, MB, Canada)

2.5 cm in diameter was used for MRI. Mice were anesthetized with 1.5 to 2% isoflurane in 30% oxygen and 70% nitrogen and placed in an animal holder with a thermal pad keeping body temperature at 37 to 37.5°C. The respiration rate was monitored using a pneumatic pillow connected to a respiratory monitor (Small Animal Instruments Inc., Stony Brook, NY, USA). Relative CBF was measured using an adiabatic spin labeling sequence with a 36 -echo half-Fourier acquisition single-shot turbo spin-echo readout following a 400 msec post tagging delay at 1 mm slice thickness and in-plane resolution of 234 μm. Slices were obtained at the plane of the injection site and at bregma for CBF measurement.

For T2 weighted images a multi-slice 8-echo rapid acquisition refocused echo (RARE) method with effective echo time TE = 80 ms and slice thickness 0.5 mm and 100 μm in-plane resolution was used. A total of 12 continuous slices were obtained with 1 mm inter-slice spacing in two 6-slice sets. MRI analysis was performed using Marevisi 7.2 analysis software (National Research Council, Winnipeg, MB, Canada). The depth of ischemia was assessed by measuring relative blood flow within a 0.9 cm diameter circular area centered on the injection site. Those values were expressed as a percentage of pre-injection CBF values. Ischemia was defined as a decrease in CBF to less than 20% of pre-injection CBF. The volume of ischemic tissue was determined by measuring the area of hyperintense pixels in T2 weighted images, multiplying by the slice thickness and summing to obtain the volume of affected area for each animal. Regions of hyperintensity on T2 weighted images were assessed as injured tissue with potential for developing into an ischemic infarct.

2.4 Statistical analysis

The first study comparing outcomes of saline and ET-1 injections to Wt and Tg littermates was analyzed by two-way analysis of variance (ANOVA). In these ANOVAs, ipsilateral and contralateral CBF and ipsilateral infarct size were outcome variables and genotype (Wt or Tg) and drug (saline or ET-1) groups were treated as between-subject factors. For ipsilateral and contralateral CBF, assessments at 4 and 48 hours were examined for group differences. Follow-up pair-wise comparisons with Bonferroni adjustment of type I error were also conducted to examine differences across genotype and drug groups. The second study comparing outcomes of Wt and Tg littermates following IP injection of caffeine 30 min prior to IC injection of ET-1 was analyzed by two-way ANOVAs (CBF) or t-test (infarct size). No animals were excluded from analysis and no mortality occurred. All p values were two-sided, and significance was set at a value of 0.05 for multiple comparisons. Statistical analyses were performed using SAS Version 9 (SAS Institute Inc, Cary, NC) or GraphPad Prism Version 5 (GraphPad Software Inc., LaJolla, CA).

3. Results

ET-1 and saline significantly decreased CBF at 4 hours post-injection. Perfusion weighted MRI was performed at 4 and 48 hours following intra-cortical injections of ET-1 or saline. Representative pseudocolor images are shown in Figure 1A. Compared to pre-injection values, ipsilateral CBF (± SEM) was decreased following injection of ET-1 or saline (Figure 2). Ipsilateral CBF at 4 hours post-injection was reduced to 36.5 ± 6.3% and 70.5 ± 9.7% in Tg and Wt saline-treated mice and to 7.3 ± 1.3% and 12.5 ± 2.0% in Tg and Wt ET-1 treated mice. Analysis of ipsilateral CBF at 4 hours indicated a significant effect of genotype ($F(1,29) = 27.4$,

$p < 0.01$), a significant drug effect ($F(1,29) = 126.1$, $p < 0.001$) and a significant genotype by drug interaction ($F(1,29) = 14.8$, $p < 0.01$). Follow-up pair-wise comparisons indicated that ET-1 produced a greater decrease in CBF than saline, and a genotype difference was observed in mice injected with saline ($p < 0.05$) but not in mice injected with ET-1. Analysis of contralateral CBF at 4 hours also indicated a significant effect of genotype ($F(1,29) = 7.01$, $p < 0.05$) and a significant drug effect ($F(1,29) = 10.6$, $p < 0.01$) as ET-1 produced a greater decrease in contralateral CBF in hENT1 Tg mice than in Wt littermates (Figure 2, inset).

Decreased CBF persisted 48 hours following injection of ET-1 but not saline. At 48 hours after injection of ET-1 the decrease in ipsilateral CBF was still evident whereas at 48 hours after injection of saline CBF was restored to pre-injection levels (Figure 3). Two-way ANOVA revealed a statistically significant effect of drug ($F (1, 29) = 50.9$, $p < 0.001$), with a significant difference between ET-1 and saline injected mice ($p < 0.05$). No significant effect of genotype was detected. The small decreases in contralateral CBF that were observed 4 hours after ET-1 injection were resolved by 48 hours (Figure 3, inset).

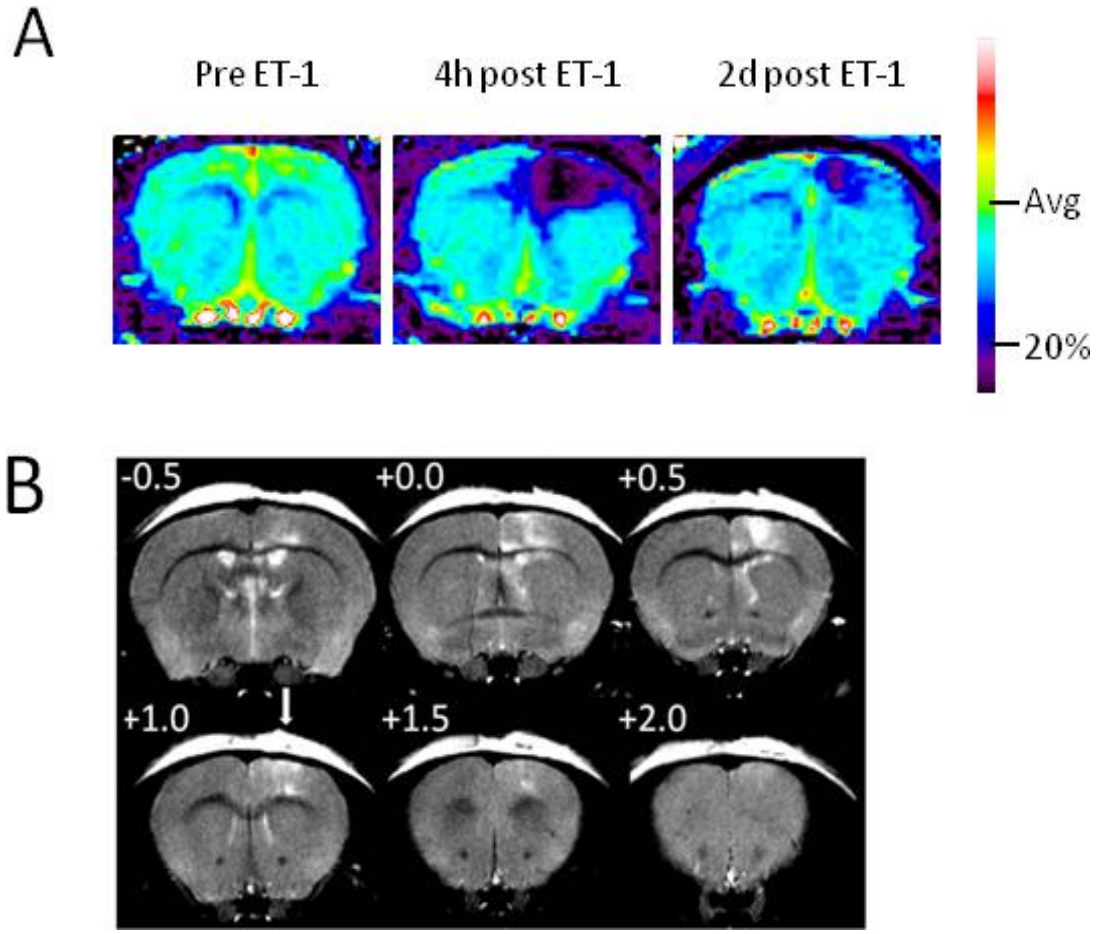
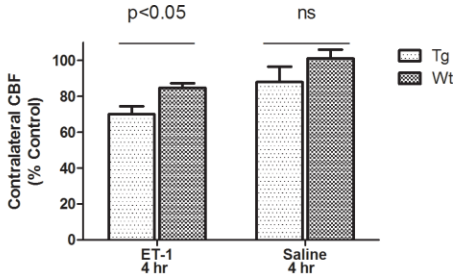


Figure 1: Perfusion weighted and T2 weighted MR images after IC injection of 1 µl ET-1.

A. Representative perfusion weighted MR images obtained from a Wt mouse before injection (pre; left) of ET-1 (400 pmol; 1 µl), and at 4 hours (4 h; middle) and 48 hours (2 d;right) post-ET-1 injection. Pseudocolor scale bar shows average CBF pre-injection (Avg) and ischemic threshold, set at 20% of pre-injection average (20%).

B. Representative T2 weighted MR images obtained from a Wt mouse 48 hours post-injection of ET-1. In each panel, the number at the upper left shows the position relative to bregma. The arrow shows the injection site at +1.0 mm anterior to bregma.

Intra-cortical ET-1 injections produced a greater ischemic size in hENT1 Tg than Wt mice. T2 weighted MRI was performed at 48 hours following intra-cortical injections of ET-1 or saline and representative images are shown in Figure 1B. Saline injections produced measurable infarcts (0.3 ± 0.1 mm^3 for Wt and 0.7 ± 0.4 mm^3 for hENT1 Tg) that were smaller than infarcts resulting from ET-1 injections (5.4 ± 0.8 mm^3 for Wt and 9.0 ± 1.1 mm^3 for hENT1 Tg) (Figure 4). Two- way ANOVA indicated a significant drug effect ($F(1, 27) = 22.3$, $p < 0.001$). Pair-wise comparisons indicated no difference between Wt and hENT1 Tg mice injected with saline but a significantly larger infarct in hENT1 Tg, relative to Wt mice, following ET-1 injection ($p < 0.05$).



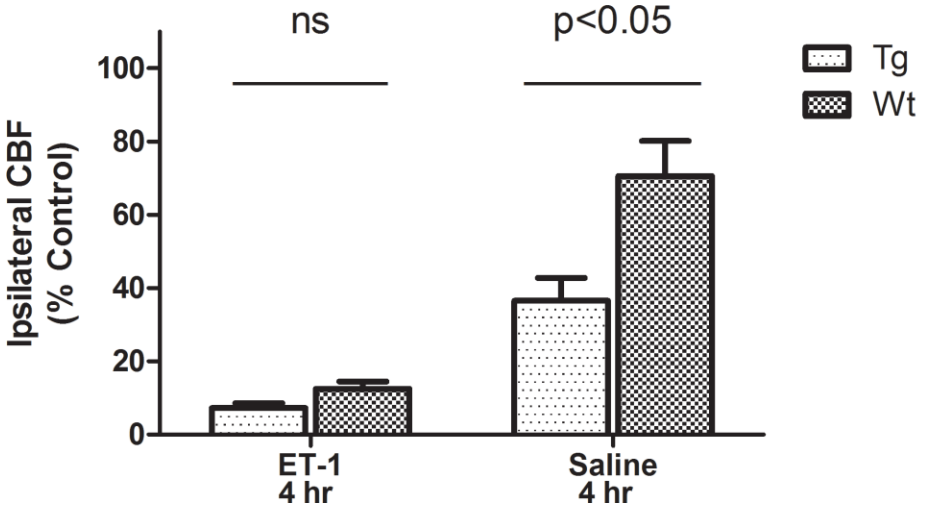


Figure 2: CBF is decreased 4 hours after IC injection of endothelin-1 (ET-1) or saline. CBF was quantitated, relative to pre-injection set at 100%, in a 0.9 cm diameter circular region-of-interest centered on the injection site. Two-way ANOVA of ipsilateral CBF indicated a significant effect of genotype ($F(1, 29) = 27.4$, $p< 0.01$), a significant drug effect ($F(1, 29) = 126.1$, $p < 0.001$) and a significant genotype by drug interaction ($F(1, 29) = 14.8$, $p < 0.01$). Bonferroni post-tests indicated a significant difference ($p < 0.05$) between Wt and Tg mice injected with saline but not between Wt and Tg mice injected with ET-1. Inset ET-1, but not saline, injections were associated with decreased contralateral CBF. Two-way ANOVA of contralateral CBF at 4 hours also indicated a significant effect of genotype ($F(1, 29) = 7.01$, $p<0.05$) and a significant drug effect ($F(1, 29) = 10.6$, $p < 0.01$). Bonferroni post-tests indicated that ET-1 produced a significantly greater decrease in contralateral CBF in Tg mice than in Wt littermates ($p < 0.05$). Data are means ± SEM, n = 12 Tg+ET-1; 13 Wt+ET-1; 4 Tg+saline and 4 Wt+saline.

3.1 Caffeine abolishes genotype differences in response to ET-1 injections

The severe and long-lasting decrease in CBF following IC injection of ET-1 was also observed in mice pre-treated with caffeine. At 4 hours following ET-1 injection, ipsilateral CBF was reduced below the ischemic threshold of 20% in both Wt and Tg mice (Figure 5A). At 48 hours after ET-1 injection, ipsilateral CBF was restored to approximately 50% of pre-ischemic levels, in both genotypes (Figure 5B). At 4 hours after ET-1 injection, contralateral CBF was reduced to 77% and 87% in Tg and Wt mice, respectively, and was restored to 93% and 99%,

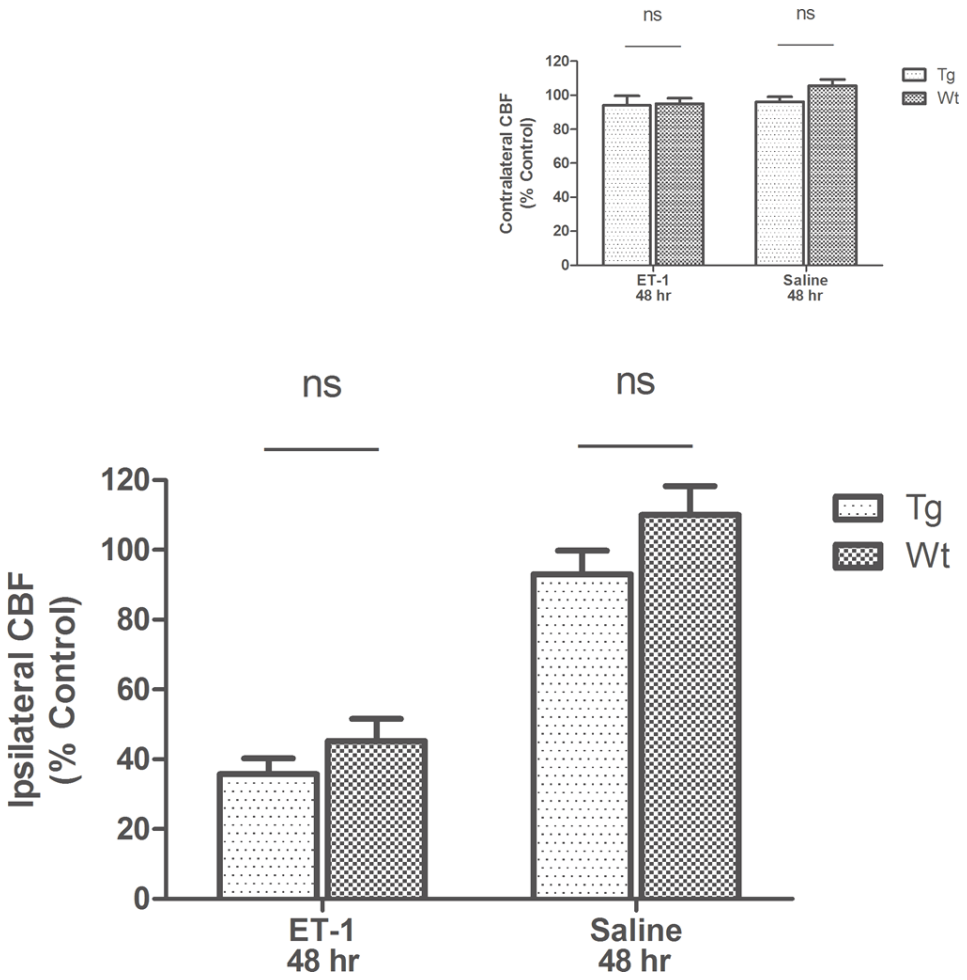


Figure 3: Decreases in ipsilateral CBF persist for 48 hours after IC injection of ET-1. CBF was quantitated in a 0.9 cm diameter circular region-of-interest centered on the injection site. Two-way ANOVA revealed a statistically significant effect of drug ($F(1, 29) = 50.9$, $p < 0.001$) and Bonferroni post-tests revealed a significant effect of ET-1 for both Wt and Tg mice ($p < 0.05$). No significant differences between Tg and Wt were detected. Contralateral CBF was restored to control levels 48 hours after injection of either saline or ET-1. Data are means ± SEM, n = 12 Tg+ET-1, 13 Wt+ET-1, 4 Tg saline and 4 Wt saline.

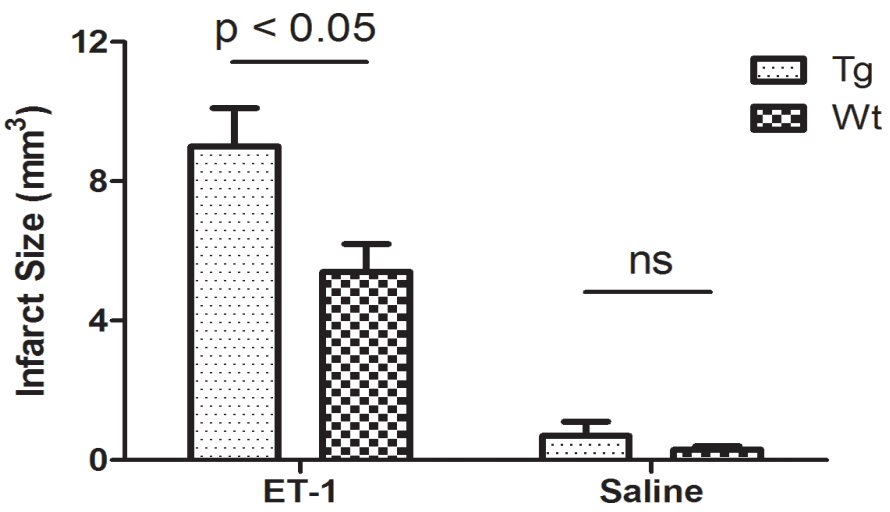


Figure 4: Ischemic injury from ET-1 injection is greater in hENT1 Tg mice compared to Wt littermates. Ischemic infarct size was determined from T2 weighted MRI at 48 hours post-injection of either saline or ET-1. Two-way ANOVA indicated a significant drug effect ($F(1, 27) = 22.3$, $p < 0.001$). Bonferroni post-tests indicated no difference between Wt and hENT1 Tg mice injected with saline but a significantly larger infarct in hENT1 Tg, relative to Wt mice, following ET-1 injection ($p < 0.05$). Data are means ± SEM, n = 12 Tg+ET-1, 13 Wt+ET-1, 4 Tg saline and 4 Wt saline.

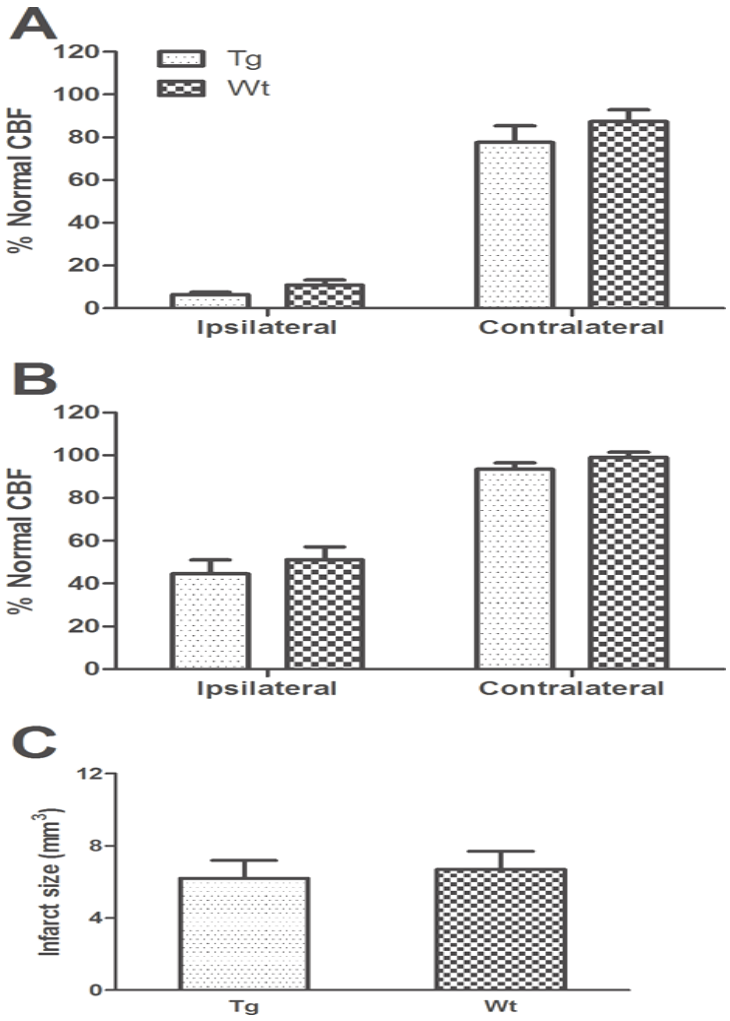


Figure 5: Caffeine pre-treatment abolished the genotype difference in severity of ET-1 induced cerebral infarcts. Mice were injected with AR antagonist caffeine (25 mg/kg, IP) 30 minutes prior to IC injection of ET-1.

A. Ipsilateral CBF was decreased to less than 20% in both Tg and Wt mice; contralateral CBF was decreased by less than 20% at 4 hours post ET-1 injection.

B. At 48 hours post ET-1 injection, partial recovery of ipsilateral CBF and full recovery of contralateral CBF was evident.

C. Ischemic infarct size, determined 48 hours after ET-1 injection was not statistically different between Tg and Wt mice. Data are means ± SEM; n =10 Tg, 10 Wt mice.

respectively, by 48 hours following ET-1 injection. These genotype differences were not statistically different. Ischemic infarct sizes, measured at 48 hours, were 6.2 ± 1 and 6.7 ± 1 mm^3, and were not significantly different between Tg and Wt mice (Figure 5C).

4. Discussion

The main finding of this study was that hENT1 Tg mice showed similar decreases in CBF, but had greater infarct sizes following ET-1 injection than Wt mice. Systemic pretreatment with the AR antagonist caffeine abolished the genotype differences in infarct size, implicating differences between Wt and hENT1 Tg mice in endogenous adenosine and AR activity.

ET-1 has been used previously to produce experimental strokes in rats (Fuxe *et al.,* 1989; Gilmour *et al.,* 2004) but different success rates have been reported with mice (Sozmen *et al.,* 2009; Wang *et al.,* 2007) leading some authors to conclude that ET-1 is not useful for mouse stroke models (Horie *et al.,* 2008; Bacigaluppi *et al.,* 2010). We found a significant CBF response to ET-1 injection that persisted for at least 48 hours ipsilaterally and led to cortical infarcts greater than 5 mm^3. Using laser Doppler to measure CBF changes in mice after IC ET-1 injection, Wang *et al.* (2007) reported an 80% drop in CBF following 1 μg ET-1 injection, similar to the 90% drop in CBF at 4 hours that we reported, and a cortical infarct of approximately 12 mm^3. Therefore we conclude that ET-1 is an effective as well as easy method for establishing a cortical infarct in mice. We did observe one limitation to the use of ET-1. We found batch to batch variability in the stroke sizes produced by ET-1 injections. The reason for batch to batch variability was not determined, but led us to ensure that all animals within an experiment had received ET-1 from the same batch. Thus, while the genotype difference in stroke size that we report here was also seen in pilot experiments, the average infarct sizes were

different with each ET-1 preparation.

It has been reported that a decrease in CBF to 20% of control levels is required for stroke injury (Siesjo, 1992). In this study, ET-1 administration produced a reduction in CBF that reached or surpassed this level of ischemia. The duration of the ischemic episode was not determined specifically, but was at least 4 hours. Using saline as a volume control, we found that mechanical or traumatic factors associated with injections produced decreases in CBF in ipsilateral cortex that persisted for 4 hours; however, these injections produced small infarcts that amounted to approximately 10% of the infarct sizes observed with injections of an equal volume of ET-1. From our data it appears that saline administration produced small cerebral infarcts without decreasing CBF below 20%. It ispossible that a significant decrease in CBF occurred but resolved within 4 hours of saline injection until our assesment with MRI.

Both saline and ET-1 produced measurable decreases in contralateral CBF; however, these decreases were modest and not of a magnitude associated with cerebral infarct. In studies using unilateral stroke models, it is sometimes assumed that the contralateral hemisphere is spared from CBF changes and that it can serve as an internal control (Villapol *et al.,* 2009). However, contralateral cell death has been observed after middle cerebral and common carotid artery occlusion to ipsilateral hemisphere (Spiegler *et al.,* 2007). Furthermore, contralateral changes in hypoxia induced factor-1α, P-Akt, P-ERK1/2 and the proinflammatory cytokines IL1-β, TNF-α and TNF-β have been observed in the absence of histopathological evidence for ischemic injury (van den Tweel *et al.,* 2006). These results all indicate that a central autoregulatory system can affect CBF responses in brain regions beyond the immediate site of injury.

Adenosine is an important neuromodulator in many neurological events including

ischemia, hypoxia and seizures (Dale and Frenguelli, 2009). During ischemic conditions, increases in adenosine levels of up to 100-fold have been measured (Parkinson *et al.,* 2000; Frenguelli *et al.,* 2007). This adenosine may be derived from neurons, astrocytes, or other cells that release ATP, which is then metabolized extracellularly to adenosine by ecto-nucleotidases. Alternatively, adenosine can be released from cells via ENT-mediated efflux, a process that has been documented in neuronal cell cultures (Zamzow *et al.,* 2008a; Parkinson and Xiong, 2004). Other mechanisms of adenosine release, including vesicular release from neurons or astrocytes have been proposed (Martin *et al.,* 2007a; Wall and Dale, 2007; Wall and Dale, 2008). In the present study, ET-1 was injected directly into a brain region that is rich in adenosine A_1Rs and expresses few A_{2A}Rs. This injection produced a similar decrease in CBF in Wt and hENT1 Tg mice at the injection site yet the resulting cerebral infarct volume was significantly greater in hENT1 Tg mice relative to Wt. This indicates that hENT1 Tg mice had reduced endogenous neuroprotection or enhanced neurotoxicity, relative to Wt mice. Using hippocampal slice preparations, we recently reported that slices from hENT1 Tg mice show increased adenosine uptake and reduced adenosine receptor activity in response to hypoxic or ischemic conditions than slices from Wt mice (Zhang *et al.,* 2011), consistent with the results of the present study. It was suggested that the brain region targeted by ET-1 injections expresses A_1Rs at a much higher density than A_{2A}Rs (Lopes *et al.,* 2004). It is likely that the effect of hENT1 expression to increase stroke injury is a direct consequence of reduced ischemia-induced increases in adenosine and decreased A_1R activation, relative to Wt mice (Zhang *et al.,* 2011). A recent study using ENT1 null mice reported decreased ischemia-reperfusion injury to heart relative to Wt controls (Rose *et al.,* 2010), which is consistent with the increased infarct size in ENT1 over-expressing mice reported here.

Caffeine is a non-selective AR antagonist with approximate Ki values of 12 and 2.4 μM for A_1 and A_{2A}Rs (Fredholm *et al.,* 1999). Caffeine was used for this study because of its clinical significance as a commonly consumed psychoactive drug. Furthermore, caffeine has been used previously in Wt and hENT1 Tg mice and affected motor activity and ethanol induced motor incoordination (Parkinson *et al.,* 2009; Kost *et al.,* 2011). The pharmacokinetics of caffeine has been examined in mice and a half-life of 25 min was reported (Buters *et al.,* 1996). Therefore, at the time of ET-1 injection, which was 30 min after caffeine treatment, therapeutic level of caffeine is expected to still be in a range at which pharmacological effects have been observed in previous studies (Parkinson *et al.,* 2009). After caffeine administration, ET-1 no longer produced a significantly greater ischemic injury in Tg mice relative to Wt mice. These data further indicate that AR activity is important for the difference in stroke injury size between Tg and Wt mice. The apparent lack of effect of caffeine in Tg mice may indicate that adenosine A_1R activity was minimal in the penumbral region in these mice, as a direct consequence of the increased neuronal uptake of adenosine mediated by functional hENT1.

5. Limitations

There were some limitations in this study. First of all, there is no histopathologic verification of infarcts identified by MRI. It is recognized that early changes in MRI after the ischemic event can result from cell swelling and reductions in extracellular space, rather than irreversibly infarcted tissue (Meng *et al.,* 2006). Second, there is no temperature control of the animals and it is well known that hypothermia is a neuroprotective treatment strategy after cerebral ischemia in animals and humans. Finally, our model used a short post-ischemic survival time and it is possible that our interventions affected the rate of infarct development rather than

final infarct size. However, it should be remembered that all animals were kept under the same conditions during and after the ischemic procedure and the environmental factors were the same for all of them.

6. Conclusion

Cortical ET-1 injection is a relatively simple and reproducible technique for producing a cerebral infarct in mice. Following ET-1 injection, Tg mice expressing hENT1 in neurons showed a greater cerebral infarct size than Wt mice. Caffeine treatment prior to ET-1 injection abolished this genotype difference, implicating AR in determining final infarct size. Our results can be explained by ENT mediated influx of adenosine into neurons and attenuated adenosine A_1R activity. This indicates that, during ischemia, neurons do not release adenosine via ENTs but actively salvage adenosine, which may be released by another mechanism, from another cell type, or formed extracellularly from released adenine nucleotides. Because, caffeine has affinity for A_1 and A_{2A}Rs as well as some degree to A_3Rs, and the cortex has a greater abundance of A_1Rs than A_{2A}Rs, future studies with an A_1R selective antagonist could enhance our understanding of role of ENT1 overexpression on AR associated neuroprotection. Beneficial role of caffeine on stroke is another interesting area of clinical research and some recent results are promising (Nehlig, 2016).

Chapter III:

Effect of the A_1 receptor antagonist DPCPX on hENT1 Tg and CD1 Wt mice in an experimental stroke model

1. Background

Adenosine is a unique nucleoside with several physiologic roles, mainly relevant to cellular energy utilization (Varma *et al.,* 2002). During pathophysiological events such as stroke or brain trauma, adenosine levels can increase up to 100-fold; the origin of this adenosine has been addressed *in vitro* using pharmacological tools but little information from *in vivo* studies is available (Parkinson *et al.,* 2000; Frenguelli *et al.,* 2007).

Adenosine can be formed from extracellular catabolism of ATP by a cascade of ecto-nucleotidases, or can be formed intracellularly from ATP consumed in energy requiring processes followed by hydrolysis of AMP by cytosolic 5'nucleotidases (Matsuoka and Ohkubo 2004). As a neuromodulator, adenosine acts through a family of metabotropic receptors which have been designated A_1, A_{2A}, A_{2B} and A_3 (Varma *et al.,* 2002; Fredholm *et al.,* 2005). Activation of adenosine A_1Rs, which are widely distributed in brain, has neuroprotective effects. In contrast, activation of A_{2A}Rs, which are most abundant in basal ganglia, can promote cell injury (Gui *et al.,* 2009). Growing evidence about adenosine in stroke raises the possibility that blocking the receptors pharmacologically, or using adenosine receptor agonists in the hours following stroke could spare patients some of the resulting disabilities (Evans *et al.,* 1987; Fricker, 2000).

ENTs transport adenosine and other nucleosides across cell membranes and mediate cellular influx or efflux as dictated by the concentration gradient (Zamzow *et al.,* 2008a). ENT1

and ENT2 mediate cellular influx or efflux of adenosine, with the direction of movement dependent upon the relative intra- and extracellular concentrations of adenosine. ENT3 and ENT4 (also known as plasma membrane monoamine transporter) are less well characterized; current evidence indicates that ENT3 is located on intracellular membranes and ENT4 has low affinity for adenosine transport (Parkinson *et al.,* 2011). The existence of multiple forms of nucleoside transporters in CNS tissue indicates a potential for development of drugs with selectivity for certain regions or cell types (Rudolphi *et al.,* 1992). Genetically altered mice can be used to investigate stroke injury at the molecular level (Horie *et al.,* 2008). Our previous *in vitro* study made use of mice with neuronal over-expression of ENT1 and reported reduced suppression of synaptic transmission and a decrease in A_1R activity in response to hypoxia and oxygen-glucose deprivation, relative to wild type littermates (Zhang *et al.,* 2011).

Standard T2-weighted MRI has been used to delineate ischemic changes in different areas of brain in animal models (Albensi *et al.,* 1999). Furthermore, measurement of perfusion status of the brain by MRI plays an important role in animal studies of cerebral ischemia. Because it is non-invasive, MRI techniques can be valuable in addressing underlying pathophysiology (Hoehn *et al.,* 2001). Of relevance, previous studies have shown that T2-weighted MRI performed two days post-injury can predict long term histopathological damage in MCA ligated rats (Peeling *et al.,* 2001).

The present *in vivo* study was performed to address three specific aims: first, to test the hypothesis that enhanced neuronal expression of ENT1 attenuates AR activation in response to an ischemic event, as was observed in our previous *in vitro* study (Zhang *et al.,* 2011); second, to test the hypothesis that DPCPX, a selective A_1R antagonist, further attenuates A_1R responses in ENT1 over-expressing mice; and third, to test for a correlation between lesion volumes

determined with T2-weighted MRI at post-operative day-2 and subsequent histopathology at day-7.

2. Materials and methods

2.1 Mice

The experiments in this study were approved by the University of Manitoba Animal Protocol Management and Review Committee. Transgenic mice with neuronal expression of hENT1 were generated on a CD1 background as previously described (Parkinson *et al.*, 2009). Heterozygous Tg male mice were bred to Wt females; 8 to 10-week-old (35-40 g) heterozygous Tg and Wt littermate male mice were used for this study. Mice were given free access to food and water and were housed in a temperature-controlled room with 12 h day/night cycles.

2.2 Pharmacological agents

Four groups of mice were studied. In each group, the study agent or vehicle was introduced by IP injection 30 min before the ET-1 administration. The first and second groups (Tg and Wt) received DPCPX (Sigma-Aldrich, Oakville, ON, Canada) in dimethyl sulfoxide (DMSO) vehicle at a dose of 1 mg/kg. The third and fourth groups (Tg and Wt) received only an equal volume of DMSO without DPCPX. DPCPX is a selective A_1R antagonist (IC_{50}: A_1 =0.69 nM, A_2 =502 nM) that is insoluble in water but soluble in DMSO (Varma *et al.* 2002). The individual who performed the experiments was blinded to genotype and identity of IP injection.

2.3 Surgical procedure

Animals were anesthetized initially with 5% isoflurane for induction then maintained with 1.5-2% isoflurane in 30% oxygen and 70% nitrogen. Rectal temperature was kept between 37-37.5 °C through a thermal pad during the procedure and was maintained by a heating lamp during the recovery. The animals were placed in a stereotaxic apparatus. Following a midline incision to the scalp, a small burr hole was performed to the right hemisphere using a microdriller. A 5 µl Hamilton syringe was used to inject 1.5 µl ET-1 (Sigma-Aldrich Canada, Oakville, ON) (1 µg ET-1 in 1µl of saline) in 7.5 min. ET-1 was always administered at 30 min after IP DPCPX or vehicle. The stereotaxic coordinates were determined from Paxinos/Franklin mouse atlas (Paxinos and Franklin, 2001) and were 1.0 mm anterior, and 1.0 mm lateral, to bregma and 1.2 mm below the pia. The needle was left *in situ* for 5 min to prevent back flow of ET-1 before being slowly withdrawn. The hole was covered by bone wax and the incision was sutured.

2.4 Magnetic resonance imaging

Animals were examined with perfusion-weighted MRI at 4 and 48 hours post-injection to confirm decreased CBF. Also they were studied with T2 weighted MRI at 48 hours to determine infarct size. A Bruker Biospec 7T/20cm spectrometer (Bruker BioSpin, Karlsruhe, Germany) with a quadrature volume coil (National Research Council, Winnipeg, MB, Canada) 2.5 cm in diameter was used for MRI. Animals were anesthetized with 1.5 to 2% isoflurane in 30% oxygen and 70% nitrogen and placed in an animal holder with a thermal pad keeping body temperature at 37 to 37.5°C. The respiration rate was monitored using a pneumatic pillow connected to a respiratory monitor (Small Animal Instruments Inc., Stony Brook, NY). Relative

CBF was measured using an adiabatic spin labeling sequence with a 36 -echo half-Fourier acquisition single-shot turbo spin-echo readout following a 400 msec post tagging delay at slice thickness 1 mm and in-plane resolution 234 µm. Slices were obtained from injection site and bregma for CBF measurement (Figure 1A, B).

For T2 weighted images a multi-slice 8-echo RARE method with effective echo time TE=80 msec and slice thickness 0.5 mm and 100 µm in-plane resolution was used. A total of 12 continuous slices were obtained with 1 mm inter-slice spacing in two 6-slice sets. MRI analysis was done by using Marevisi 7.2 analysis software (National Research Council, Winnipeg, MB). Lowest CBF was assessed by measuring a relative blood flow within 0.9 cm diameter circular area centered on the injection site. Those values were compared to our pre-injection CBF values to get percentage of decreased CBF. Ischemic volume was determined by measuring the area with less than 20% of pre-injection CBF in both slices and multiplying by 1 mm, the inter-slice spacing. Infarct size was obtained by measuring the area of hyperintense pixels in T2 weighted images, multiplying by the slice thickness and summing to obtain the infarct volume for each animal (Figure 1C).

2.5 Histopathology

Mice were deeply anesthetized with isoflurane in an anesthesia chamber and chest wall was opened when mice showed no response to a painful stimulus. The right atrium was perforated and the animal was perfused with 20 ml of 10% buffered formalin solution. The brain was removed, fixed for up to 1 week, and maintained at 4°C in the same fixative. The cerebrum was cut into 40 µm slices with a razor blade and dehydrated in a sequence of alcohol and xylene, and embedded in paraffin. Sections at each level were cut and stained with hematoxylin and

eosin stain (H&E) for histopathologic examination. Using a charge coupled device camera (Olympus, Japan) all areas showing necrosis or neuronal loss were marked through an image software program (Image J, NIH). The final values were the volume of the affected area and it was sum of all values (by Cavalieri principle) as measured by using Image J (Figure 1D).

2.6 Statistical analysis

Volumes of reduced blood flow, determined from perfusion weighted MRI, volumes of cerebral infarct, determined from T2 weighted MRI, and volumes of cerebral infarct, determined from histopathology, were compared using two-tailed t-test. Comparisons of infarct size between four groups were analyzed by one way ANOVA with Tukey's post-test. Values of $p<0.05$ were considered statistically significant.

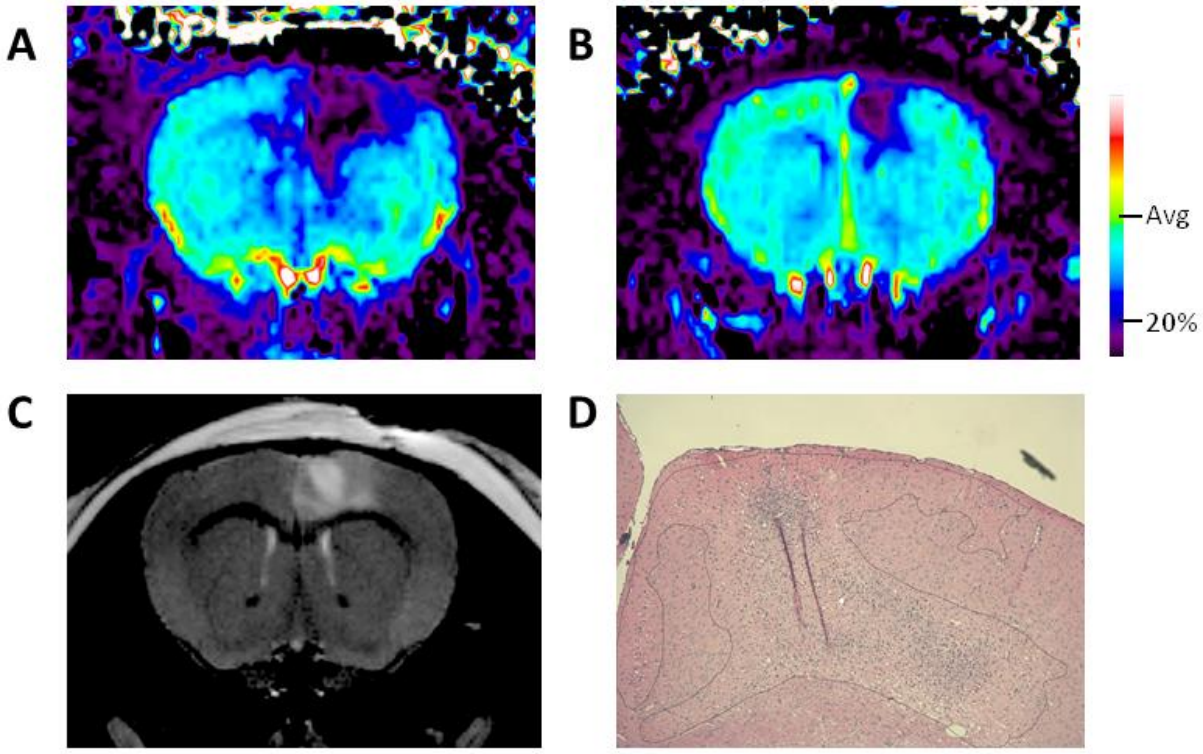


Figure 1: Perfusion weighted and T2 weighted MRI images and histopathology after IC injection of 1.5 µl (600 pmol) ET-1 in Tg mouse brain.

A.Representative perfusion weighted MRI image at 4 hours post ET-1 injection. Ischemic area extends from cortical area to upper striatum.

B. Representative perfusion weighted MRI image at 48 hours post ET-1 injection. The region with reduced CBF is largely restricted to the cortex. Pseudocolor scale bar shows average CBF pre-injection (average) and ischemic threshold, set at 20% of pre-injection average.

C. Representative T2 weighted MRI image obtained 48 hours post-injection of ET-1. Note the hyperintense area of motor cortex in Tg mouse.

D. Histopathological image (H&E) of the same area as in C obtained at 7 days post-injection of ET-1. A necrotic core area is outlined and the surrounding penumbra is shown.

3. Results

3.1 Cerebral blood flow changes at 4 and 48 hours after IC ET-1 injection

As illustrated in Figure 2, compared to pre-injection values, at 4 hours after cortical ET-1 injection CBF (± SEM) was significantly decreased to 12.0 ± 1.3% (n = 20) and 11.3 ± 1.9% (n=20) at the injection site compared to contralateral site, which was 78.1 ± 4.5% and 75.6 ± 5.8% in DPCPX and vehicle groups, respectively ($p<0.001$, Figure 2). There was no difference between DPCPX and vehicle groups in 4 hour CBF values at the injection and contralateral sites ($p>0.05$).

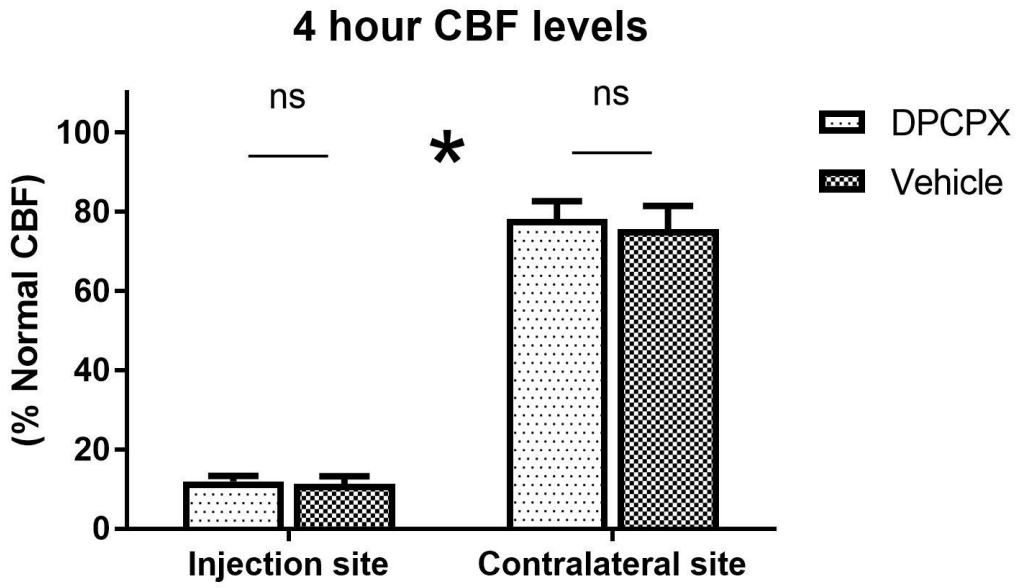


Figure 2: CBF at 4 hours after IC injection of ET-1. CBF was quantitated, relative to pre-injection set at 100%, in a 0.9 mm diameter circular region-of-interest centered on the injection site. CBF was decreased both ipsilateral and contralateral to the injection site. Both DPCPX and vehicle (DMSO) show similar drop in CBF in either site ($p>0.05$). However, compared to their contralateral sites, ET-1 injection showed a significant drop in CBF at the injection site in both DPCPX and vehicle groups ($p<0.001$) (Indicated with * between two bars comparing the difference between injection and contralateral sites)

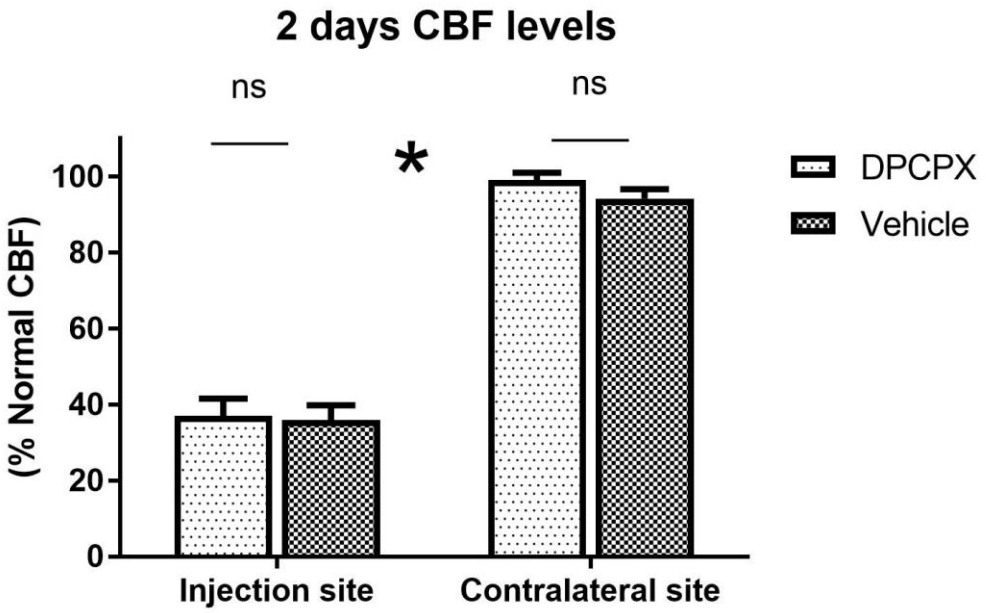


Figure 3: CBF at 48 hours following ET-1 injection. CBF was increased, relative to 4 hours after ET-1 injection, in both sides and contralateral blood flow was restored to pre-injection levels. Note similar CBF levels in both DPCPX and vehicle groups ($p>0.05$). Compared to their contralateral hemispheres, injection sites (injection site) still show a significant drop in CBF in both DPCPX and vehicle groups ($p<0.001$). (Indicated with * between two bars comparing injection and contralateral sites)

At 48 hours, CBF showed some improvement. Ipsilaterally, CBF increased to 37.1 ± 4.5% of control in DPCPX and to 35.9 ± 3.9% of control in vehicle treated mice ($p>0.05$), Contralaterally, CBF increased to 99 ± 2% and 94.2 ± 2.48% of control in DPCPX and vehicle

treated mice, respectively ($p>0.05$). Similar to 4 hours post-injection, there was no difference between DPCPX and vehicle groups on either side for 48 hours CBF values ($p>0.05$), but the difference in CBF between ipsilateral and contralateral site was highly significant ($p<0.001$) (Figure 3).

3.2. Transgenic mice showed lower CBF at 4 hours but not at 48 hours

Compared to their Wt littermates (n=10), Tg mice (n=10) showed significantly lower CBF at the injection site at 4 hours in DPCPX group (15.4 ± 1.6% vs. 8.5 ± 1.5%, respectively, $p=0.04$) and in vehicle group (n=10 Wt, 10 Tg) (16.6 ± 2.6% vs. 6 ± 1.4%, $p=0.01$) (Figure 4A). Cerebral blood flow at the contralateral site showed no significant difference between Tg and Wt mice. At 48 hours, CBF improved in all groups and the difference between Wt and Tg mice decreased in DPCPX group (43.3 ± 3.6% vs. 30.9 ± 7.9%, respectively, $p>0.05$) and disappeared in vehicle group (36 ± 4.9% vs. 35.9 ± 6.4%, $p>0.05$) (Figure 4B).

3.3 Intracortical ET-1 injections produced a greater infarct size in DPCPX group than vehicle group

T2 weighted images obtained 48 hours after injection of ET-1 were obtained. Administration of DPCPX caused a greater infarct size on MRI compared to vehicle group (5.03 ± 0.8 mm^3 vs. 4.24 ± 0.6 mm^3, respectively); similar results were obtained with analysis of histopathology (5.43 ± 1 mm^3 vs. 3.65 ± 0.6 mm^3). However, all these changes were not statistically significant ($p>0.05$) (Figure 5A).

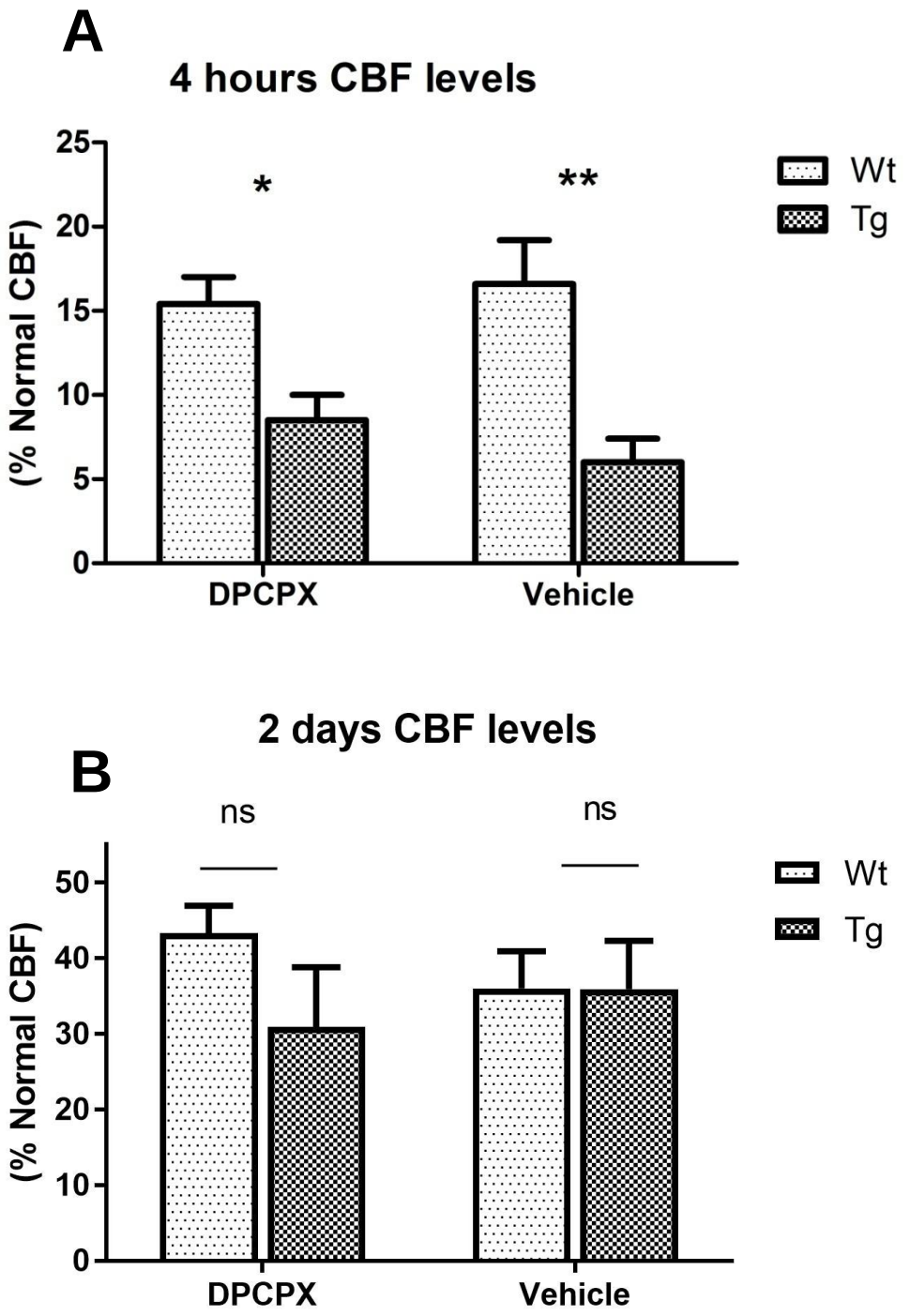


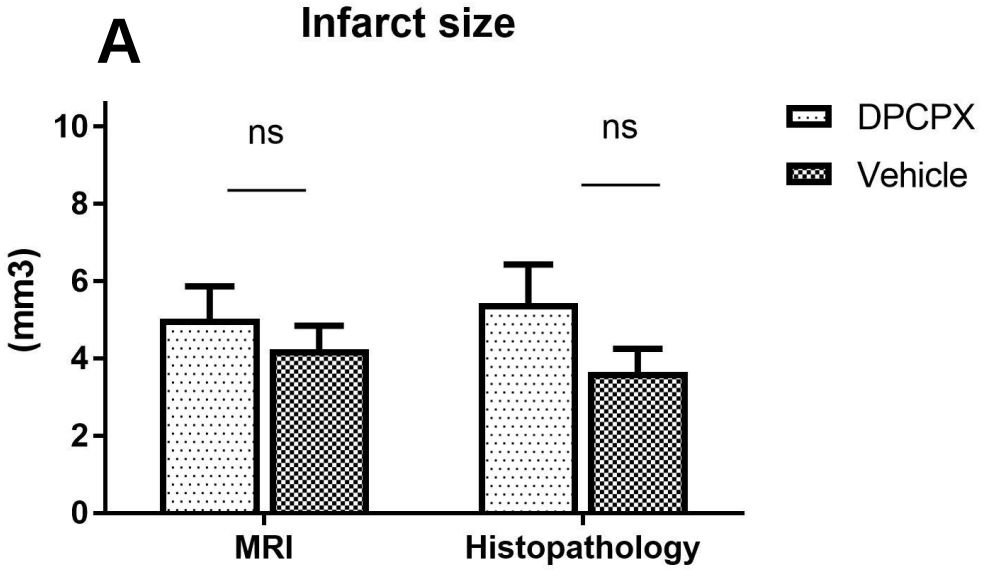
Figure 4: CBF at 4 hours and 48 hours following ET-1 injection in hENT1 Tg and Wt mice from ipsilateral (injection) site. Contralateral site was not shown because of almost normal CBF.

A. Transgenic mice show significantly lower CBF values than Wt mice at the injection site in DPCPX-treated (15.4 ± 1.6% vs. 8.5 ± 1.5% respectively, *p<0.05) and in vehicle treated (16.6 ± 2.6% vs. 6 ± 1.4%, **p<0.01) groups.

B. At 48 hours following ET-1 injection, CBF shows improvement. Tg mice continue to have lower CBF than Wt mice in DPCPX treated groups, but this was not statistically significant (p>0.05). Vehicle treated mice have almost identical CBF regardless to their genotype (p>0.05) (ns= not significant).

3.4 Intracortical ET-1 injections produced a greater infarct size in Tg than in Wt mice especially in DPCPX group on histopathology

Compared to Wt littermates, Tg mice treated with DPCPX had greater infarct sizes on MRI (3.97 ± 0.7 mm^3 vs. 6.09 ± 1.4 mm^3, respectively), although this did not reach statistical significance (p>0.05). In vehicle treated groups, infarct size was greater in Tg than in Wt mice (4.49 ± 1 mm^3 vs 3.98 ± 0.5 mm^3) but this was not statistically significant (p>0.05). However, the results from histopathology showed a statistically significant increase in infarct size in DPCPX-treated Tg (7.29 ± 1.72 mm^3) compared to vehicle-treated Wt mice (2.9 ± 0.52 mm^3) p<0.05) (Figure 5B).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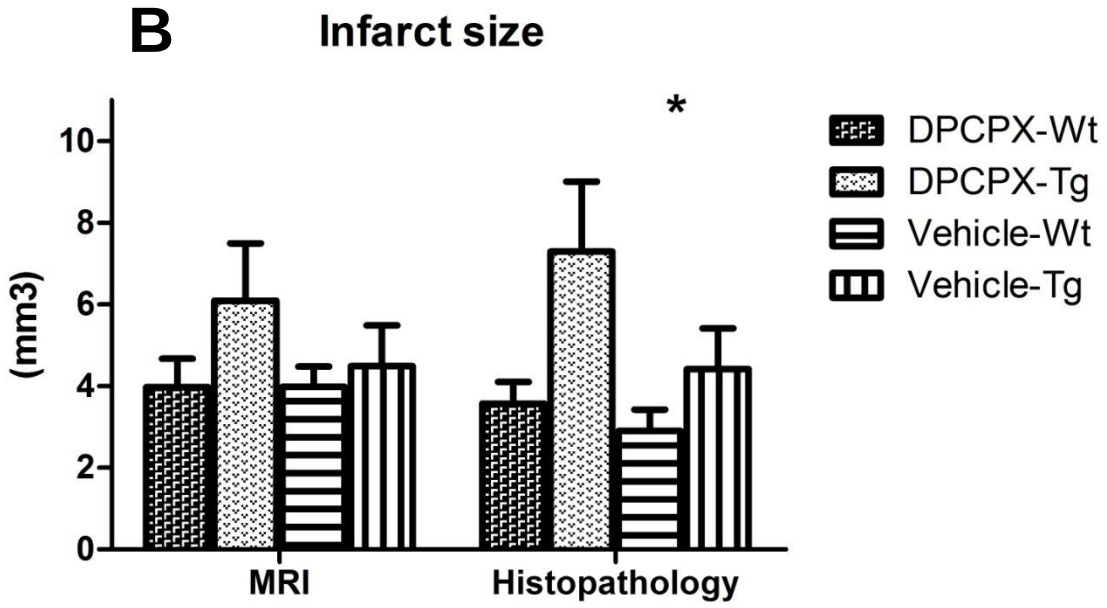
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Figure 5. Cerebral infarct volumes determined by MRI and by histopathology at 2 days and 7 days, respectively, following ET-1 injection.

A. MRI (48 hours) and histopathology (7 days) studies show greater ischemic injury after ET-1 injection in DPCPX-treated than in vehicle-treated mice but the difference is not statistically significant (n=20, $p>0.05$) (ns= not significant).

B. hENT1 Tg mice show a trend towards a larger injury compared to Wt littermates by both MRI and histopathology in both DPCPX-treated and vehicle-treated groups. This finding was statistically significant by histopathology analysis in Tg group that received DPCPX, relative to vehicle treated Wt. Data are means ± SEM, $*p < 0.05$, one way ANOVA with Tukey's post-test.

4. Discussion

In this study, mice that over expressed ENT1 were used to show its regulatory role on extracellular adenosine concentrations in a cortical stroke model. In addition, A_1R was targeted since it is the most abundant AR in neocortex and neuroprotective. Among the 4 subtypes of ENTs, ENT1 and ENT2 are widely expressed in CNS (Chen *et al.*, 2007; Anderson *et al.*, 1999). Current study has provided additional evidence that over expression of ENT1 is detrimental for stroke injury. ET-1 caused a significant decrease in CBF in Tg mice which was mostly correlated with subsequent larger cortical damage. The results were consistent with our previous electrophysiological and *in vivo* studies, and showed ENT1 over expression in hypoxic/ischemic conditions is detrimental for neurons. That indicates decreased adenosine receptor activity and

increased adenosine uptake (Zhang *et al.,* 2011). It is a well known fact that adenosine is an important neuromodulator in many neurological events including ischemia, hypoxia, and seizures (Dale and Frenguelli, 2009). During ischemic conditions, adenosine levels increase up to 100-fold (Frenguelli *et al.,* 2007; Parkinson *et al.* 2000). To date, the common thought was that extracellular adenosine rises in parallel with intracellular adenosine, which is the main source of adenosine, through nonconcentrative bidirectional NTs. (Fredholm *et al.,* 2005). However, in integrated brain preparations, ENT1 blockage did not decrease but increased extracellular adenosine concentrations, which indicates its role could be mostly clearing of adenosine from extracellular environment, opposite to common thought (Cunha *et al.,* 2005). The exact mechanism of this inward concentration gradient of adenosine during ischemic events is not clear yet. Wall and Dale (2008) have speculated intracellular AK activity also helps to keep the free intracellular adenosine concentration low. But AK activity could also be detrimental as Pignataro *et al.* (2007) showed that up to three times larger infarct size occurs in AK Tg mice that over express AK. In our laboratory, we have shown that astrocytes might contribute to increase extracellular adenosine concentrations (Zamzow *et al.,* 2008b; Parkinson *et al.,* 2005). Therefore, ENT1 has a crucial role to regulate adenosine levels, which can result in neuroprotection or neurotoxicity. We suggest that ENT1 drives adenosine influx during hypoxia and the result is decreased extracellular adenosine concentrations and increased risk for neurotoxicity. Indeed, lower activity or inhibition of ENT1 was neuroprotective (Chen *et al.* (2007; Cui *et al.,* 2013; Nguyen *et al.,* 2015) but hyperactivity was neuro detrimental (Zhang *et al.,* 2011). In some other reports, ENT1 acts to reduce levels of extracellular adenosine in the amygdala and that absence or inhibition of ENT1 elevates extracellular adenosine in that brain region (Chen *et al.,* 2007). Similar to this observation a recent study using ENT1 null mice

reported a decrease in ischemia-reperfusion injury in heart relative to Wt controls (Rose *et al.,* 2010). We also previously showed that locomotor activity in response to caffeine administration is significantly decreased in hENT1 Tg mice compared to Wt littermates (Kost *et al.,* 2011). In that study Tg mice were shown to have 9.6-13.7 fold increase in ENT1. All these results together with our current study strongly suggest a detrimental role for ENT1 over expression or hyperactivity through decreasing extracellular adenosine concentrations under stressful or hypoxic/ischemic conditions and indicates that ENT1 could be a pharmacological target for cerebral insults in the future.

Adenosine produces its effect through a family of four G-protein coupled receptors, termed A_1, A_{2A}, A_{2B}, and A_3Rs. In brain, these receptors are located on neurons, astrocytes, microglia, and endothelial cells (Dunwiddie and Masino, 2001; Fredholm *et al.,* 2005). A_1Rs and A_{2A}Rs show non-uniform distribution in brain. Fastbom *et al.* (1987) showed that mouse temporal and occipital cortex (Layers I-VI) have A_1R density around 43 to 72% relative to the density in the stratum radiatum of the CA1 hippocampal area, which has a high density of A_1Rs. In contrast, A_{2A}Rs are predominantly on neurons in the striatum, nucleus accumbens and olfactory tubercle (Cunha *et al.,* 1994). In the present study, our target was to establish a cortical infarct at the fronto-parietal cortex, which affects the motor functions and is rich in A_1Rs. Therefore, we have used A_1R specific antagonist, DPCPX to see whether the effects of A_1R blockage in this specific area would alter adenosine related neuroprotection. Although, A_1R blockage caused a larger infarct size, the difference did not reach statistical significance when Tg and Wt mice combined. But, when it was separated as Tg and Wt, DPCPX caused a significantly larger infarct in Tg on histopathology compared to others. This can be explained by genotype because there was no similar significance between Wt animals that both received DPCPX. Thus,

the infarct size should be at least partially from A_1R blockage. We explain this finding by synergistic neurotoxicity of Tg genotype and A_1R blockage. However, we should remember that DPCPX was delivered in DMSO and DMSO is considered as a neuroactive perhaps light neuroprotective agent. Paunescu et al., (2009) reported analgesic effect of DMSO in mice. Our *in vivo* results supports our recent *in vitro* study, which shows that inhibitory effects of adenosine on synaptic activity were reversed by 1μM DPCPX in hippocampal slices (Zhang *et al.,* 2011). However, it should be noted that every brain region has different abundance of ARs with different roles and the function of adenosine is receptor related (Sebastiao and Ribeiro, 2009). In the present study we chose 1 mg/kg DPCPX given IP 30 min prior to ET-1 injection. The dose was considered optimal for an A_1R related experiment. Phillis (1995) compared 0.1 mg/kg and 1 mg/kg of DPCPX in a gerbil study and 1 mg/kg showed a significant increase in ischemia-induced damage in hippocampus. However, Prediger *et al*. (2004) showed no effect of DPCPX at 3 mg/kg, but an anxiogenic-like response developed at 6 mg/kg on in the elevated plus-maze in mice. This could imply that the optimal dose of DPCPX could show variations from one animal to other. But overall, we observed detrimental effect of A_1R blockage via its antagonist DPCPX and this is not surprising by considering neuroprotective role of A_1Rs in cortex. Varma *et al.* (2002) compared adenosine A_1R agonist and antagonists in a controlled cortical impact model in mice. They found DPCPX at 12 nM exacerbates volume of damaged area significantly compared to sham group but DMSO vehicle alone showed almost the same effect as DPCPX in that experiment. DMSO activity on A_1Rs was also reported by Varma *et al.* (2002). This help us to explain similarities in CBF at 4 and 48 hours between DPCPX and vehicle groups. Overall, the results were very consistent with the literature and our previous studies and ENT1 over expression resulted in neurotoxicity and A_1R blockage exaggerated this injury. Unfortunately,

there is no better alternative to use as a vehicle for DPCPX since another vehicle, ethanol, is also a neuro-active substance.

In addition, adenosine receptors can form heteromers, for example A_1/A_{2A}Rs heteromers, producing complexes with opposing functions (A_1 versus A_{2A}) (Boison, 2008). Besides abundant A_1Rs in neocortex, Cunha showed functional A_{2A}Rs in blood vessels (Cunha, 2005). Although, we did not include A_{2A}Rs in our study because our target area, cortex, is abundant in A_1Rs, but there is still a chance that a vasodilatory effect of A_{2A}Rs could abolish the difference. It is possible that DPCPX, by blocking A_1Rs, increases the amount of adenosine available to act on A_{2A}Rs and this could produce similar CBF and infarct size in DPCPX and vehicle treated groups when genotype was ignored. If we compare current results with previous experiment, caffeine has abolished genotype effect but DPCPX failed to do it. Caffeine is non-selective AR antagonist however DPCPX is A_1R specific antagonist. This also support other AR's potential beneficial role, beside of A_1Rs, in neuroprotection from hypoxia/ischemia.

Another purpose of this study was to demonstrate a simple and relatively non-invasive technique in establishing stroke by direct injection of ET-1 to the cortex and measuring subsequent ischemic changes by using MRI and histopathology. We have shown this technique was effective and easy as was documented before (Soylu *et al*., 2012). Intracortical ET-1 injection technique is more popular in rats than in mice, since more consistent stroke size was observed in rats. However, compared to other methods, such as distal MCA occlusion or thromboembolic models, ET-1 can reduce blood supply selectively in frontoparietal cortex and induces an infarct involving all layers of the neocortex (Bacigaluppi *et al.,* 2010, Fuxe *et al.,* 1997). Moreover, ET-1 is not directly neurotoxic but produces a temporary loss of blood flow for several hours at the injection site (Windle *et al.,* 2006; Luo and Grammas, 2010). Markus (2003)

showed that if CBF is kept above 21-23 ml/100 mg/min, then infarction does not occur. But if it is reduced below 6-10 ml/100 mg/min for more than a very brief period, then infarction always occurs, even if blood flow is then restored. Seisjo (1992) showed that a decrease in CBF to 20% of control levels is required for stroke injury. We observed a decrease of CBF under 20% of basal levels in all ET-1 injected groups so far. Indeed, CBF has dropped to 10% of normal levels around 4 hours and slowly restored to 30% in 48 hours. Changes in CBF were strong enough to produce a moderate sized cortical infarct which was evident by T2 weighted MRI and histopathology. Dose of ET-1 was sufficient. Wang *et al.* (2007) reported 80% decrease in CBF following 1 µg ET-1 injection by using laser Doppler method in mice, which is close to our values (Wang *et al.,* 2007).

5. Limitations

There were some limitations in our study. First of all, DPCPX needs either ethyl alcohol or DMSO as a vehicle for *in vivo* use. However, both substances have side effects on neurological system. Therefore, DMSO was chosen as a better option in our experiment compared to alcohol, but its effect in CNS should be considered in interpretation of the results. In addition, there was no temperature control of the animals. Although we kept all animals in the same temperature throughout and after the experiment, it should be remembered that hypothermia is a neuroprotective factor and it could affect the injury size when it happens.

6. Conclusion

Our results showed again that enhanced neuronal expression of ENT1 can be neurodetrimental and can elevate neuronal damage during hypoxia/ischemia. This was

statistically significant when combination of Tg mice and blockage of A_1R receptors by DPCPX happened. This indicates a synergistic role of overexpressed hENT1 which deprives adenosine from extracellular environment and blockage of neuroprotective A_1Rs by antagonist DPCPX increases magnitude of this damage. This is consistent with our previous results and the literature. However, DPCPX did not make any statistical difference compared to the vehicle when genotype was ignored and the groups were combined, but we know that DMSO is also neuro-active agent and is reported in some studies as neuroprotective. Therefore, failure of DPCPX to establish larger stroke size in combined groups could be vehicle, DMSO, related. Our finding could help us to better understand this novel role for ENT1 and could make it a target for preventing stroke/ischemia related conditions in the future. Our previous finding about abolished genotype effect by caffeine was not reproduced with the selective A_1R antagonist. Therefore, we suggest that other ARs could also play a role in cortical hypoxa/ischemia. In addition, our methods for establishing cortical stroke by using ET-1 in mice and detecting CBF and injured area by MRI are simple and relatively non-invasive ways for animal research using genetically modified mice as a stroke model. We also showed that histopathology is very useful tool for determining magnitude of neuronal injury and is closely correlated with MRI or *vice versa*.

Chapter IV:

Effect of caffeine intake during pregnancy on AR ontogeny and caffeine metabolism in newborn rats [2]

1. Background

Caffeine is the most widely consumed psychoactive drug in the world by adults (Leon *et al.*, 2002). Nearly 90% of US adults consume caffeine in forms of coffee, tea or other caffeinated products (Yang *et al.*, 2010). The majority of expectant mothers in western society drink caffeinated beverages during pregnancy and they continue their consumption during lactation (Aden, 2011). Average caffeine intake is estimated to be 164 mg/day among women 18-34 years and 125 mg/day among pregnant women although it could be quite variable among individuals (Browne, 2006). Metabolism of caffeine is progressively impaired from first to third trimester in pregnant women, leading to accumulating levels in fetuses and neonates (Aden, 2011; Okubo *et al.*, 2015). In a recent study, it was reported that the half life of caffeine increased from 4.71 hours in non-pregnant women to 15.08 hours in pregnant women (Hahn *et al.* 2015).

Caffeine readily passes through the placenta and blood-brain barriers and enters all tissues (Aden, 2011). Since the main enzyme (CYP 1A2) is absent in both placenta and the fetus, caffeine accumulates in fetal tissues (Chen *et al.*, 2014). McGowan *et al*. (1988) described caffeine-associated withdrawal symptoms around 1 day of life with vomiting, intermittent bradycardia, periodic breathing, apnea, tachypnea and jitteriness in infants born to mothers with high caffeine intake during pregnancy.

[2] All WB and rt-PCR experiments were performed by Wei Xiong. All HPLC analysis was performed by Casey Sayre.

Chronic caffeine exposure of fetuses is associated with low birth weight (LBW) and risk of premature birth (Leon *et al.,* 2002). In addition, a recent systematic review revealed that excessive caffeine intake during pregnancy is associated with an increase in spontaneous abortion, stillbirth, low birth weight, and small for gestational age, but not preterm delivery (Greenwood *et al.,* 2014). Animal studies with chronic caffeine treatment produced conflicting results: both increased and decreased brain injury have been reported (Gray *et al.,* 2011; Schmidt *et al.,* 2006; Stevenson, 2007).

During long-term administration of caffeine, many functions of the organism develop tolerance, including cardiovascular system and CNS and changes in both functionality and gene expression of adenosine receptors have been postulated to be responsible for tolerance to the effects of caffeine following long-term caffeine treatment. Tolerance appears more marked at high doses of caffeine than at low doses (Leon *et al.,* 2002). Up-regulation of A_1Rs after chronic adenosine receptor antagonism occurs, but A_{2A}R levels apparently do not change (Ribeiro and Sebastiao, 2010). The increased expression of A_1Rs in response to chronic antagonism of ARs by caffeine, as compared with A_{2A}Rs, may lead to a shift in the A_1/A_{2A}R balance after prolonged caffeine intake (Ribeiro and Sebastiao, 2010). The consequences of these receptor changes on animals such as behavior, apnea frequency, neuroprotective or neurodetrimental effects are controversial (Leon *et al.,* 2002). However, it should be remembered that adrenergic, cholinergic, GABA and serotonin receptors are also affected from caffeine especially in higher doses (Brent *et al.,* 2011).

In many caffeine and pregnancy related studies, the dose of caffeine and its effect on outcome shows remarkable variations. In this study, we aimed to see if high dose of caffeine consumption affects AR ontogeny in the offspring. We believe this could be important since

caffeine consumption during pregnancy is very common and ARs are targets for caffeine. Any change in this ontogeny could affect quality of the response to caffeine treatment postnatally. The qualitative response to caffeine is also very important because caffeine is one of the commonest medications in newborn medicine. Therefore, we designed a rat study to examine postnatal consequences of prenatal caffeine exposure.

2. Objective/Hypotheses:

The objectives of this study were to use an animal model (a) to evaluate the effect of caffeine treatment on pregnancy outcomes; and (b) to assess the effect of intrauterine exposure to caffeine on abundance and expression of A_1 and A_{2A}Rs in rat offspring. Our hypotheses were (a) high dose caffeine during pregnancy will affect neonatal outcomes such as litter size and birth weight, and (b) caffeine consumption during pregnancy will change AR ontogeny since it crosses placental and blood-brain barriers almost completely.

3. Methodology:

All animal protocols were performed in accordance with the guidelines detailed by the Canadian Council on Animal Care and were approved by the University of Manitoba Animal Care Committee. Sprague Dawley rats were kept in a ventilated room under controlled conditions of lighting and optimal temperature (21°C, 12:12-hour dark-light cycle, with lights on at 08:00 and off at 20:00) with free access to food and plain or caffeinated drinking water. Rats were mated and when pregnancy was confirmed by an animal care technician, female rats were assigned to one of two groups. The first group (designated caffeine group; (Cg)) received 1 g/l of caffeine citrate (equal to 0.5 g/l of caffeine base) in drinking water from gestation day 1

throughout the entire gestation period. The second group (designated water group; (Wg)) included control rats supplied with caffeine-free tap water (Figure 1). Caffeine and water bottles were refreshed every other day. All animals were weighed every second day. On the day of delivery, caffeine supplementation was terminated and all animals were provided with tap water. Postnatal 1st day (P0) and postnatal 7th day (P6) pups were sacrificed for experimental use.

After birth, both groups were further stratified into 2 subgroups. Male and female pups were used in equal numbers. The first subgroup was sacrificed on P0 and brain tissue was collected for WB and rt-PCR analysis of A_1R and $A_{2A}R$ protein and mRNA expression. The second subgroup received caffeine citrate IP at 60 mg/kg/dose (equal to 30 mg/kg/dose of caffeine base) on P6 and caffeine metabolism study finished within 120 min. Additional rats were also sacrificed on P6 and brain samples were collected and analyzed by WB and rt-PCR for A_1R and $A_{2A}R$.

3.1 Demographical and pregnancy outcome measures:

During pregnancy, water consumption, caffeine intake, and weight gain was measured. The number of pups in each litter and the weight of each pup at P0 and P6 was also recorded.

3.2 Determination of brain A_1 and $A_{2A}R$ density in cortex, striatum and BS by WB analysis

For WB analysis, brain cortex, striatum and BS of rat pups on P0 and P6 were dissected and stored separately at -80C until the study was performed.

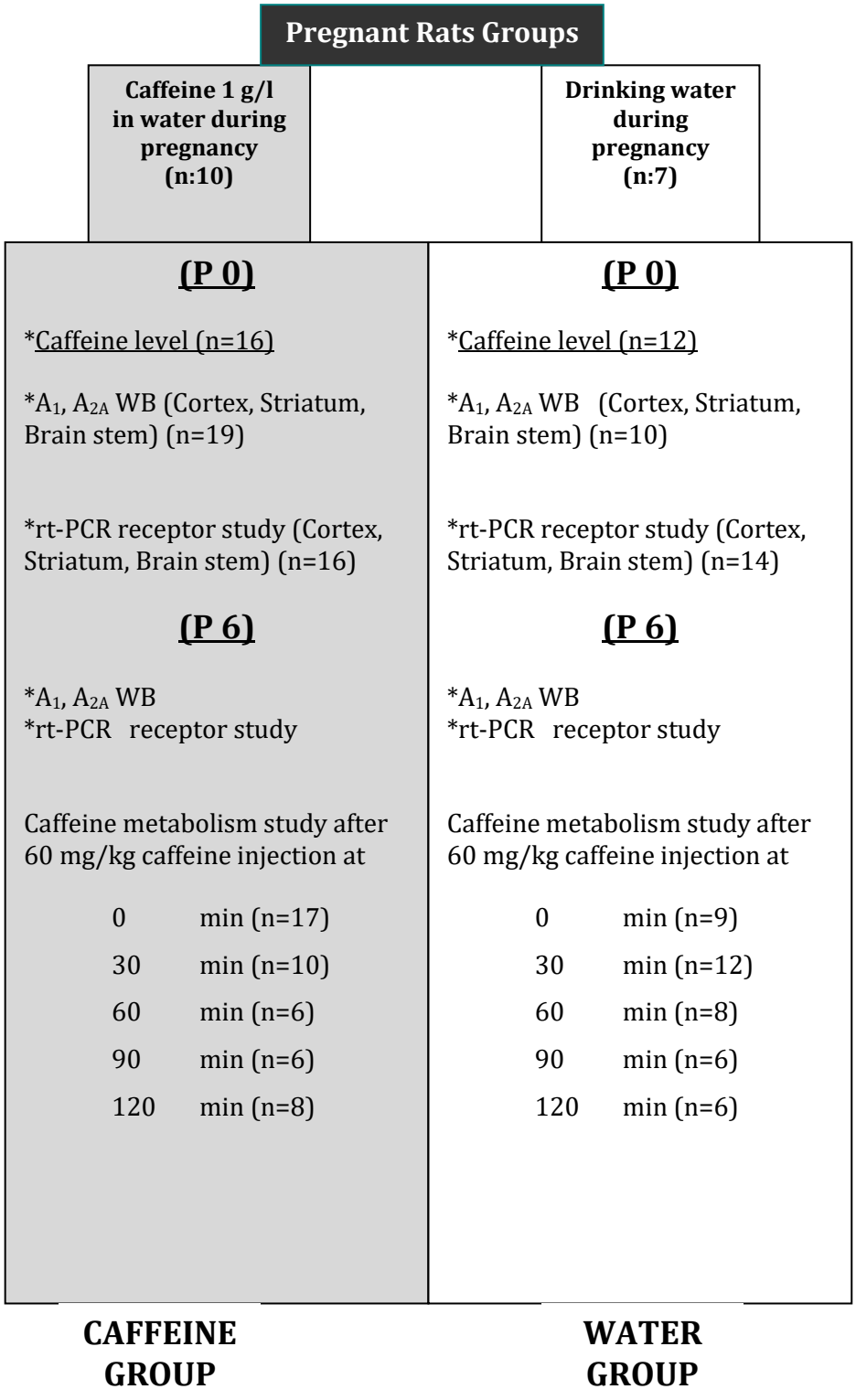

Figure 1: Study design is summarized on the flow chart

Samples in RIPA buffer (150mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH:8.0) were sonicated for 20 seconds on ice. 50-100 µg of proteins were separated on 10% SDS-PAGE, and transferred onto PVDF membranes. The electroblotted membranes were incubated with primary antibody overnight at 4ºC. Subsequently, membranes were incubated with secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized on ChemiDoc MP System by an enhanced chemiluminiscence detection kit. Protein content was determined with the Bio-Rad protein dye binding reagent. Protein loading was confirmed with actin antibody. For A_1R, a rabbit polyclonal antibody was purchased from ThermoFisher Scientific (PA1-041A). For $A_{2A}R$, a rabbit polyclonal antibody was purchased from ThermoFisher Scientific (PA1-042). For actin, a goat polyclonal antibody was purchased from Santa Cruz Biotechnology Inc (sc-1616). Secondary antibodies were horseradish peroxidase conjugated goat anti-rabbit (Bio-Rad Laboratories; 170-6515) or horseradish peroxidase conjugated donkey anti-goat (Santa Cruz Biotechnology Inc; sc-2020).

3.3 Determination of brain A_1 and $A_{2A}R$ expression in cortex, striatum and BS by rt-PCR

For rt- PCR, 2.0 µg total RNA from each sample was treated with DNase I according to Invitrogen DNase I treatment protocol (http://tools.lifetechnologies.com/content/sfs/manuals/purelink_micro_midi_rna_man.pdf). We used following primers for the analysis: rA_1-310: CTCCATTCTGGCTCTGCTCG, rA_1-516: ACACTGCCGTTGGCTCTCC, rA_{2A}-441: CCATGCTGGGCTGGAACA, rA_{2A}-590: GAAGGGGCAGTAACACGAACG. RNA samples were reverse transcribed using oligo-dT 12-18 primer to produce cDNA. 1 µl (of 60 µl) of the resulting cDNA was used for rt-PCR reaction.

The rt-PCR was run in 25 µl volume and its cycle for all primer pairs consisted of 50 cycles at 95°C for 15 seconds (s), 64°C for 15 s, 72°C for 30 s and melt curve in a Bio-Rad iCycler iQ RT-PCR Detection System in 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5µM primer, 0.1 x SYBR Green I, 0.25 unit platinum Taq polymerase, 10 nM fluorescein standard.

3.4 Caffeine levels by HPLC after 60 mg/kg loading dose to P6 rat pups

To test for an effect of intrauterine caffeine exposure on post-natal caffeine metabolism, P6 pups were administered 60 mg/kg of caffeine citrate by IP injection. The pups were anesthetized with isoflurane and intracardiac blood was collected from untreated rats and from rats 30, 60, 90 or 120 min post-injection. The blood samples were centrifuged for 10 min at 20,000 RPM and plasma was collected and stored at -80 °C.

For high performance liquid chromatography analysis of plasma caffeine levels, the limit of quantitation was 0.5 µg/ml and the limit of detection was 0.1 µg/ml at a wavelength of 274nm. The mobile phase was 30:70 methanol: H_2O with a run time of 20 min. Retention time of caffeine was 10.5 min. Antipyrine was used as an internal standard and had a retention time of 15.4 min.

3.5 Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD). The difference in demographical data between 2 groups was analyzed by unpaired two-tailed student-t test. Statistical analyses were performed using statistical package for the social sciences (SPSS) software program (SPSS Version 20, USA). A p-value of 0.05 was considered statistically significant. Graphs were prepared using the Graphpad Prism program (Version 5.04, USA).

4. Results

4.1 Demographic Rresults

Seventeen Sprague-Dawley rats (10 in Cg and 7 in plain Wg) and their pups were used for this study. By providing 1 g/l of caffeine citrate supplement in drinking water, rats received an average of 92 ±31 mg/kg/day caffeine citrate which equals to 46 ± 16 mg/kg/day of caffeine base during the pregnancy (Table 1).

There was a difference in daily water consumption between Cg and Wg. Average daily water consumption in Cg was significantly less than Wg during pregnancy, 91.7±30.8 ml/kg/day vs. 126.3±29.24 ml/kg/day ($p < 0.05$) (Table 1).

There was also a difference in weight gain during pregnancy. Mothers in Cg gained 6.8±1.46 g each day during pregnancy. Their average weight increased from 302.3±34.28 g to 439.5±33.29 g through pregnancy (Table 1). Mothers in Wg gained 8.5±1.13 g each day during pregnancy. Their average weight increased from 319.1±27.37 g to 489.8±19.58 g through pregnancy (Table 1). The difference for weight gain between 2 groups was significant ($p<0.05$).

Rats in Cg delivered significantly fewer pups per mother than rats in Wg: 12.1±4.28 vs. 18.9±1.07 pups, respectively ($p<0.01$) (Table 1). In addition, the average birth weight of pups in Cg was significantly lower than in Wg: 5.79±0.54 g vs. 6.31±0.33 g, respectively ($p< 0.0001$) (Table 1).

Interestingly, the difference in weights between the 2 groups was no longer statistically significant by P6. However, Wg remained slightly bigger than Cg in average weight: 17.68±2.64 g vs.16.61±2.04 g, respectively ($p>0.05$) (Table 1).

Table 1: Pregnancy and post-partum features

PREGNANCY AND POST-PARTUM FEATURES		
Pregnany Outcomes	**Caffeine Group (n=10)**	**Water Group (n=7)**
Average water intake/day	92 ± 31ml/kg/day	126 ± 30 ml/kg/day*
Average caffeine intake (as caffeine citrate)	92 ± 31 mg/kg/day	------
Average weight gain/day	6.8 ± 1.5 g/day	8.5 ± 1.1 g/day*
Average pups number	12 ± 4	19 ± 1*
Postpartum Outcomes	**Caffeine Group**	**Water Group**
Female/Male Ratio (P0)	0.93	1.07
Average weight (P0)	5.8 ± 0.5 g	6.3 ± 0.3g*
Female/Male Ratio (P6)	1	1
Average weight (P6)	17.7 ± 2.6 g	16.6 ± 2 g
Serum caffeine level (P0)	5.32 ±2.03 µg/ml	0.21 ± 0.07 µg/ml

*$p < 0.05$

4.2 Adenosine receptor ontogeny:

By WB analysis, A_1R was detected as a band of approximately 37 kD. A_1R expression did not show any significant difference in cortex (Figure 2) or BS (Figure 4) of P0 pups in the caffeine or Wg. However, A_1R protein expression in the striatum was significantly higher in Cg than Wg (7.06 ± 0.55 vs. 5.01 ± 0.64, p = 0.025) (Figure 3). This difference was not present in P6 rats (Figure 5).

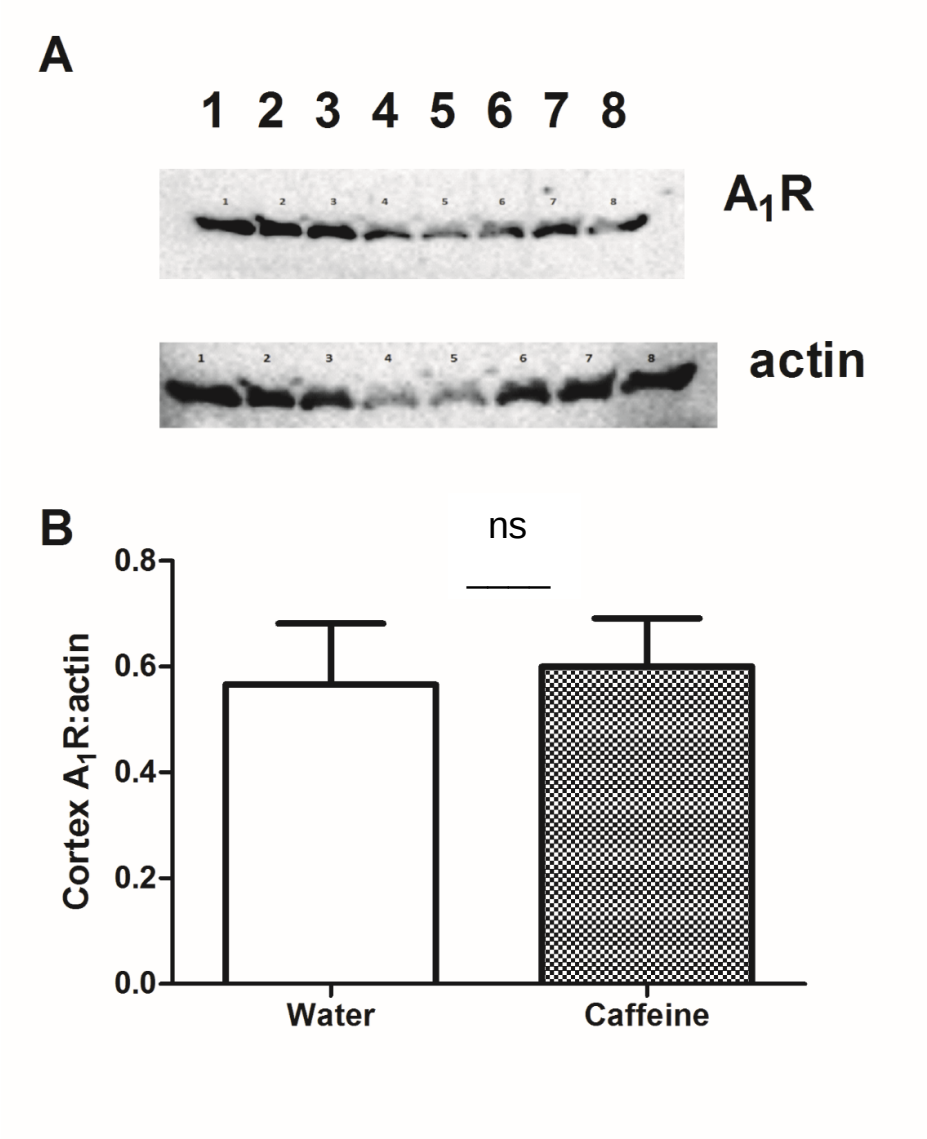

Figure 2: WB analysis for A_1R expression in the cortex of P0 pups.

A. A representative WB for A_1R (upper blot) shows a band at approximately 45 kD. Blots were stripped and reprobed for actin (lower blot), which was detected as a band at approximately 40 kD. Lanes 1-4 are samples from Wg and lanes 5-8 are from Cg.

B. Band intensity for A_1R was normalized to actin band intensity and no significant difference in A_1R between groups was detected. Values (± SE) were 0.57 ± 0.12 for Wg (n:10) and 0.60 ± 0.09 for Cg (n:19) ($p>0.05$).

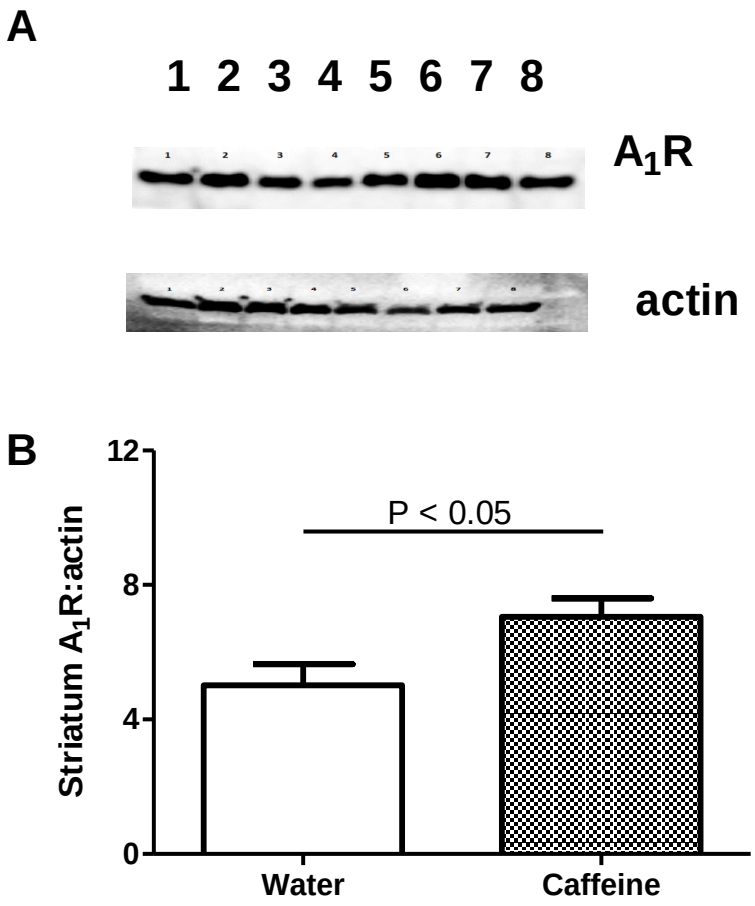

Figure 3: WB analysis for A_1R expression in the striatum of P0 pups.

A. A representative WB for A_1R (upper blot) shows a band at approximately 45 kD. Blots were stripped and reprobed for actin (lower blot), which was detected as a band at approximately 40 kD. Lanes 1-4 are samples from Wg and lanes 5-8 are from Cg.

B. Band intensity for A_1R was normalized to actin band intensity. A_1R protein expression was significantly higher in Cg (n:19; 7.06 ± 0.55) than Wg (n:10; 5.01 ± 0.64) ($p = 0.025$).

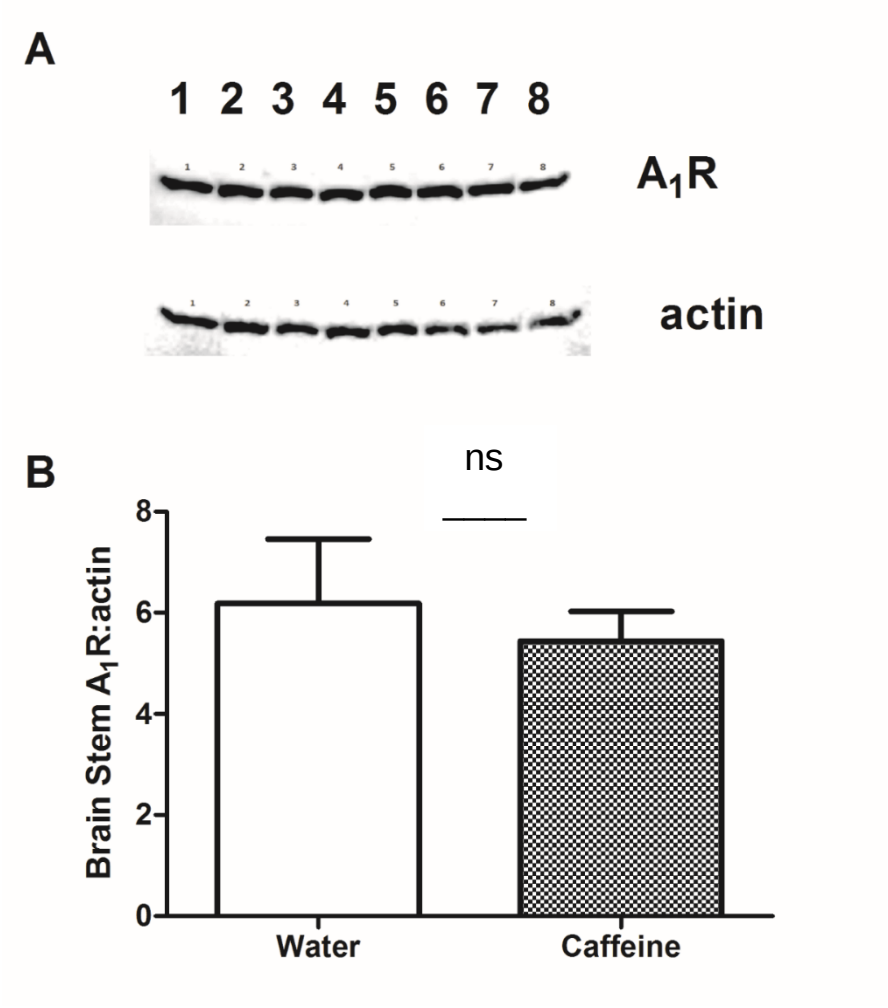

Figure 4: WB analysis for A_1R expression in the BS of P0 pups.

A. A representative WB for A_1R (upper blot) shows a band at approximately 45 kD. Blots were stripped and reprobed for actin (lower blot), which was detected as a band at approximately 40 kD. Lanes 1-4 are samples from Wg and lanes 5-8 are from Cg.

B. Band intensity for A_1R was normalized to actin band intensity. A_1R protein expression did not show any significant difference in BS of P0 rat pups between two groups as a result of caffeine intake during pregnancy. Values were 6.18 ± 1.27 (Water) and 5.44 ± 0.58 (Cafffeine)($p>0.05$).

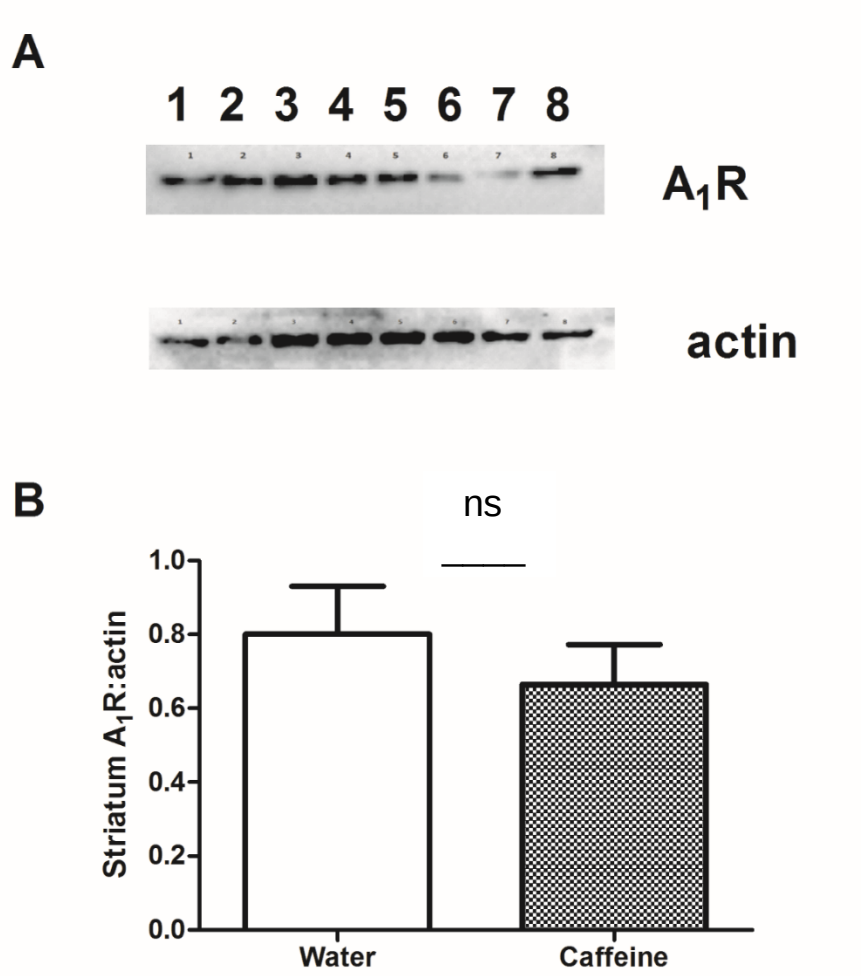

Figure 5: WB analysis for A_1R expression in the striatum of P6 pups.

A. A representative WB for A_1R (upper blot) shows a band at approximately 45 kD. Blots were stripped and reprobed for actin (lower blot), which was detected as a band at approximately 40 kD. Lanes 1-4 are samples from Wg and lanes 5-8 are from Cg.

B. Band intensity for A_1R was normalized to actin band intensity. A_1R protein expression was similar in Wg and Cg (0.80 ± 0.13 vs. 0.67 ± 0.11, respectively, $p > 0.05$).

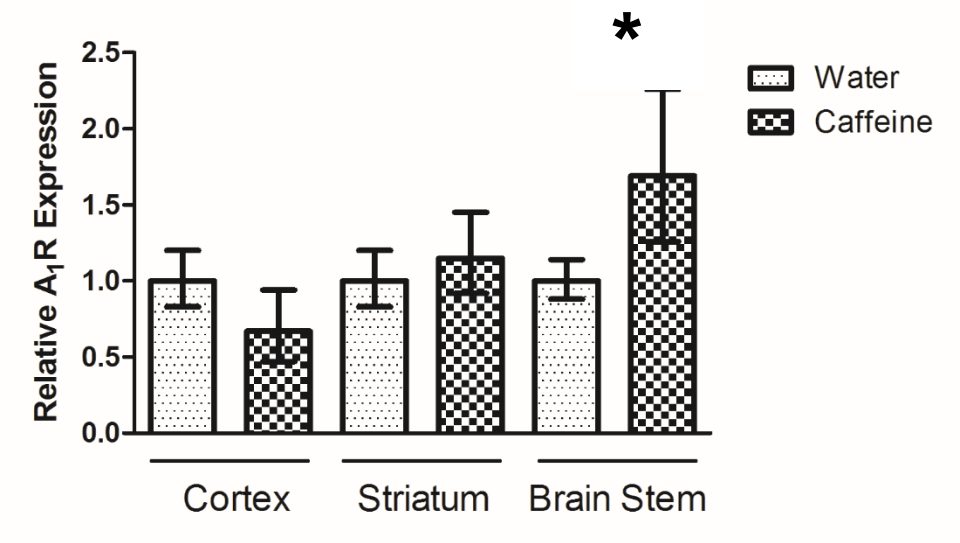

Figure 6: rt-PCR analysis for A_1R expression in the cortex, striatum and BS of P0 pups. Total RNA was isolated from brain regions, reverse transcribed and amplified by rt-PCR. A_1R expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and values from Cg are expressed relative to the Wg. Values (95% C.I.) for Wg (n:12) and Cg (n:16) were 1.0 (0.83-1.20) and 0.67 (0.47-0.94) for cortex, 1.0 (0.83-1.20) and 1.15 (0.92-1.45) for striatum and 1.0 (0.88-1.14) and 1.69 (1.26-2.26) for BS (*$p<0.05$).

Although in brain tissue from adult rat, the $A_{2A}R$ antibody detected a single protein at 45 kD, there was no detectable $A_{2A}R$ protein expression in any brain region by WB analysis in P0 pups. Therefore, P6 study was not performed.

By rt-PCR there was no significant difference in A_1R expression in cortex or striatum but BS showed a significant increase (CI 95%, 1.26-2.26) in the Cg relative to the Wg in P0 pups (Figure 6). Similarly, there was no significant difference in $A_{2A}R$ expression in P0 pups in the

cortex or striatum but the BS showed a significant increase in $A_{2A}R$ expression (CI 95%, 3.09-5.67) in the Cg (Figure 7; $p<0.05$). BS rt-PCR was performed in P6 rats for the follow up. The increased expression of A_1R and $A_{2A}R$ was no longer evident by day 7 in the BS (Figure 8). The other two areas were not studied.

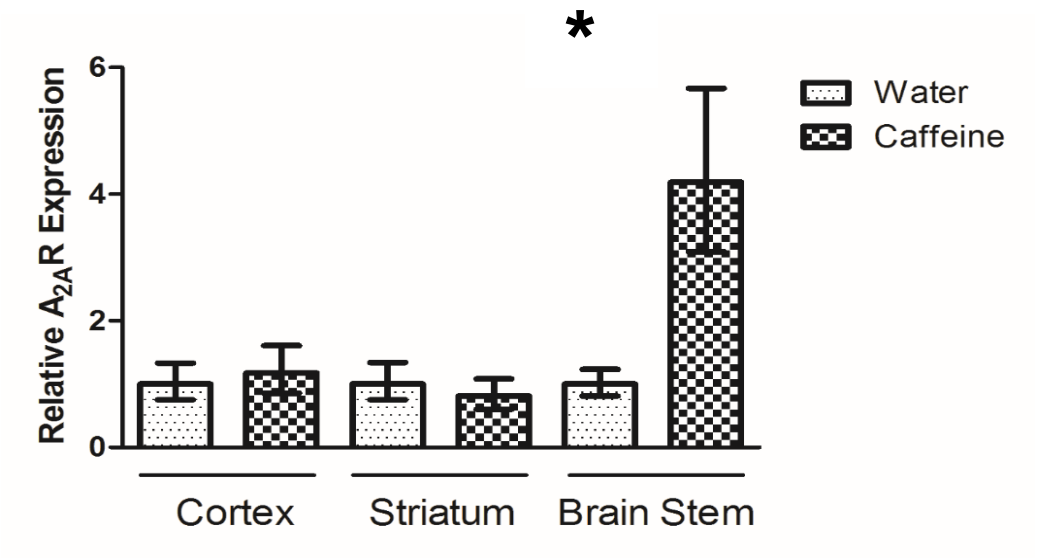

Figure 7: rt-PCR analysis for $A_{2A}R$ expression in the cortex, striatum and BS of P0 pups. Total RNA was isolated from brain regions, reverse transcribed and amplified by rt-PCR. $A_{2A}R$ expression was normalized to GAPDH and values from Cg are expressed relative to the Wg. Values (95% C.I.) for Wg and Cg were 1.0 (0.75-1.33) and 1.18 (0.86-1.61) for cortex, 1.0 (0.75-1.34) and 0.81 (0.60-1.08) for striatum and 1.0 (0.81-1.23) and 4.19 (3.09-5.67) for BS (*$p<0.05$)

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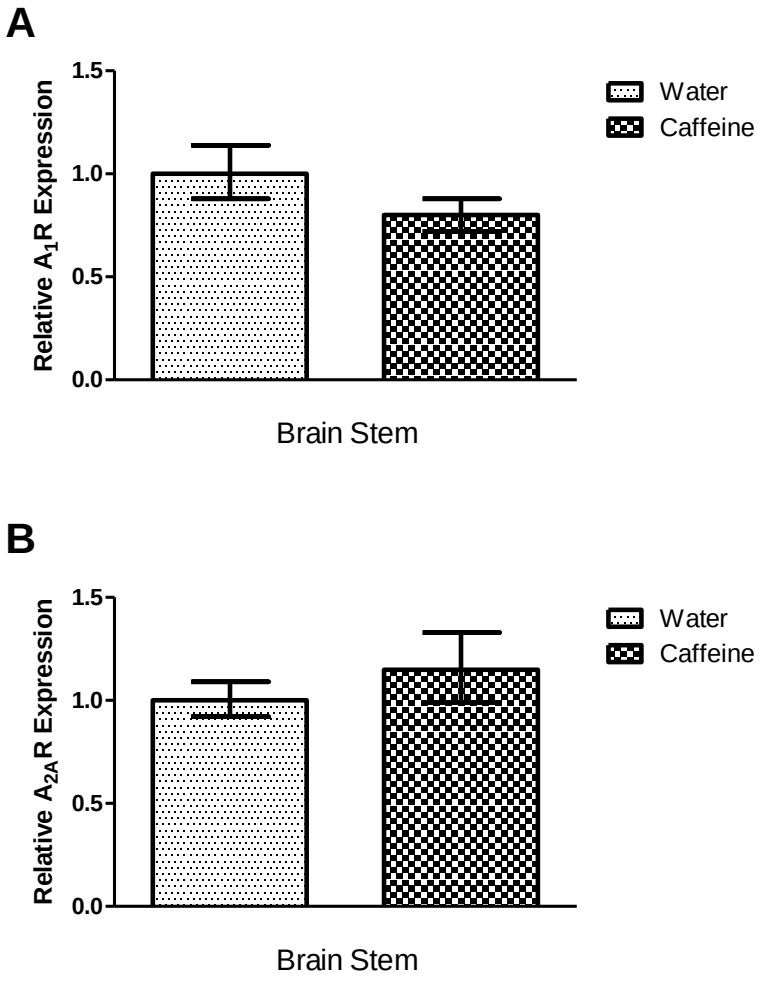


Figure 8: rt-PCR analysis for A_1R and $A_{2A}R$ expression in the BS of P6 pups. Total RNA was isolated from brain regions, reverse transcribed and amplified by rt-PCR. A_1R or $A_{2A}R$ expression was normalized to GAPDH and values from Cg are expressed relative to the Wg.

A. A_1R expression. Values (95% C.I.) for Wg and Cg were were 1.0 (0.88-1.14) and 0.80 (0.72-0.88).

B. $A_{2A}R$ expression. Values (95% C.I.) were 1.0 (0.92-1.09) and 1.15 (0.99-1.33).

4.3 Caffeine metabolism

By using high performance liquid chromatography, serum caffeine levels were analysed in P0 rat pups. As expected, there was a very significant difference ($p<0.0001$) in average (± SD) serum levels of caffeine between pups from mothers treated with caffeine during pregnancy (5.32 ±2.03 µg/ml) vs. pups from mothers given tap water (0.21 ± 0.07 µg/ml) (Figure 9).

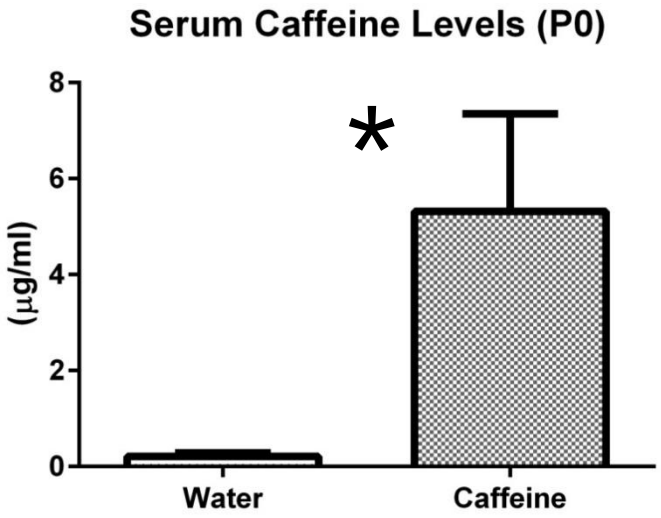


Figure 9: Caffeine levels in serum of P0 rat pups by HPLC. There was a significant difference in average plasma caffeine levels (± SD) between rats born to caffeine supplied mothers 5.32 ± 2.03 µg/ml) vs. plain water supplied mothers (0.21 ± 0.07 µg/ml, *$p<0.0001$).

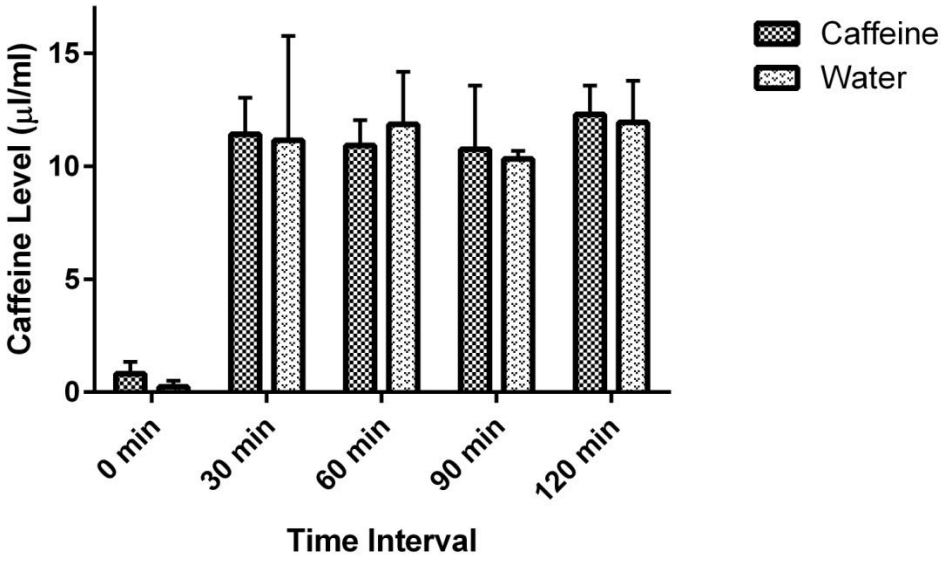


Figure 10: Serum caffeine levels following 60 mg/kg IP caffeine citrate in P6 rat pups by HPLC. There was a baseline caffeine level at 0 min in rats born to caffeine supplied mothers (0.82 ± 0.52 µg/ml) vs. plain water supplied mothers (0.24 ± 0.27 µg/ml, $p>0.05$). It was followed by a peak in both groups at 30 min after the injection (11.41 ± 1.62 vs. 11.14 ± 4.62 µg/ml respectively, $p>0.05$), and continued as a plateau at 60 min (10.92 ± 1.11 vs. 11.85 ± 2.32 µg/ml respectively, $p>0.05$), at 90 min (10.75 ± 2.82 vs. 10.32 ± 0.35 µg/ml

Plasma caffeine levels were measured in P6 rats after IP injection of 60 mg/kg caffeine citrate. Peak plasma levels were observed at 30 min post-injection and no significant differences were observed up to 120 min post-injection. No differences in plasma caffeine levels were observed between rats from caffeine and water treatment groups (Figure 10).

5. Discussion:

5.1 Caffeine metabolism and concentration during pregnancy and in other situations

Caffeine is rapidly and completely absorbed from the gastrointestinal tract with minimal first-pass effect and enters all body tissues by freely crossing the blood-brain, placental and blood-testicular barriers (Grosso *et al.,* 2006). In rat, the effect of pregnancy on the pharmacokinetics of caffeine is not known because of large individual variations in pregnant rats (Nehlig and Debry, 1994). In humans, consumption of 2–3 cups of coffee provides 250 mg of caffeine and is equal to 3.5 mg/kg/day in a 70 kg average weight person (Leon *et al.,* 2002). However, a caffeine dose in humans vs. rats is not equal, and there is a conversion factor of 1/6.17 between humans and rats (Luo *et al.,* 2014). By using this conversion factor, our dose of 92 mg/kg/day caffeine citrate (46 mg/kg/day of caffeine base) in rats was equivalent to 14.9 mg/kg/day caffeine citrate (7.5 mg/kg/day of caffeine base) in humans. In our study caffeine levels on the first day of life was 5.32 μg/ml. It has been reported that the therapeutic window of plasma caffeine concentration is 5-25 μg/ml in newborn humans (Aden, 2011). Therefore, we propose the dose used in our study, 1 g/l caffeine supplementation, achieved an optimal therapeutic level of caffeine in newborn pups and it seems a reasonable dose for caffeine studies in rats. However, it is higher than the dose used in most of the studies in the literature and the achieved plasma concentrations are different among the studies and animals. Previously, the same concentration of caffeine in drinking water yielded an 83 mg/kg/day intake and 10 μg/ml of plasma caffeine concentration (Lorenzo *et al.,* 2010). In another study by Shi *et al.* (1994) 3 days of caffeine supplementation at 1 g/l in adult mice provided 100 mg/kg/day intake and 2.1 μg/ml of plasma caffeine concentration. Picard *et al.* (2008) used 0.2 g/l caffeine supplementation throughout whole pregnancy in rats, and a dose of 46 mg/kg/day provided 1.25 μg/ml in their P0

pups. These results indicate that plasma caffeine concentrations show variabilities according to animal species and strains, dose and duration of supplementation, and age of the animals. These differences in plasma caffeine levels may explain the different results observed following similar supplement doses.

5.2 Caffeine metabolism in newborn rats after the exposure during pregnancy and/or neonatal period

Caffeine is rapidly and totally absorbed from gastrointestinal tract and there is very little first-pass effect. It enters all tissues passing blood-brain and placenta barriers (Aden, 2011). The kinetics in rats is dose dependent and zero order, indicating a saturable process, especially at higher doses (Brent *et al.,* 2011). The major metabolic difference between rodents and humans is that, in the rat, 40% of caffeine metabolites are trimethyl derivatives whereas these represent only 6% in humans (Nehlig and Debry, 1994). A cytochrome P450 enzyme, Cyp 1A2, demethylates caffeine into dimethylxanthine metabolites (Yang *et al.,* 2010). Another aim in our study was to elucidate if maternal caffeine intake during pregnancy affects neonatal caffeine metabolism. In current literature there is very limited information to answer this question. We observed a reliable level of plasma caffeine in P0 pups (5.32 ±2.03 µg/ml) by exposure to 92 ±31 mg/kg/day caffeine during gestation. However, there was no effect of this exposure on rate of caffeine metabolism in P6 rats. Plasma caffeine levels were similar in the two groups of pups after 60 mg/kg of caffeine citrate on postnatal day 6. In clinical practice, newborn babies show variable responses to the same dose of caffeine. Based on the results from the present study, we suggest this variability in responses in human babies is not caused by changes in caffeine metabolism due to previous (*in utero*) exposure. However, our results need to be supported with

further studies addressing the levels of caffeine eliminating enzymes, especially CYP 1A2, to understand whether any change occurs at the molecular level.

5.3 Effect of caffeine on pregnancy outcomes

We found a significant decrease in offspring numbers in rats receiving a high dose of caffeine during pregnancy. Although caffeine`s effect on fertility in humans has been discussed over 30 years, the results are inconclusive (Aden, 2011; Okubo *et al.*, 2015). Animal data do not answer this question clearly. Adverse effects of caffeine on pup numbers were reported only in a few studies. Fujii and Nishimura (1972) supplied pregnant rats with a mixed diet that contains 0.50% caffeine by weight during whole pregnancy period. They compared this with 0.25% caffeine by weight during whole pregnancy or with standard diet. They found a 38.3% resorption rate in fetuses in high dose group and 17.4% in low dose and 6.5% in control group. The average litter size was 7.9 g, 11.6 g and 13.3 g in these groups, respectively. Results of their study are highly supportive for our observation of a detrimental effect of caffeine on litter size. However there is no clear explanation about its mechanism. Kren *et al.* (2004) showed a detrimental effect of caffeine on oocyte maturation in cell culture. Sullivan (1988) reported that a single dose of caffeine at 60 mg/l is more teratogenic than 200 mg/l caffeine that was given in the diet or drinking water. Therefore, we believe detrimental effect of caffeine is multifactorial and is affected by caffeine protocol and stage or duration of pregnancy that was caffeine-exposed. Otherwise, Aden *et al*. (2000) showed that caffeine ingestion during the first week of pregnancy did not alter fecundity (10.88 ± 0.55 pups and 10.91 ± 0.59 pups, for caffeine-exposed and control animals, respectively). However, human data is more extensive than animal studies on this issue. Christianson *et al.* (1989) reported that caffeine consumption of >7 cups/day is

associated with almost twice the risk for decreased fertility. Very shortly after this study, Joesoef *et al.* (1990) reported that there is no such relation between caffeine and fertility. Pre-conceptional caffeine intake and spontaneous abortion was investigated by Hahn *et al.* (2015). They found an association between late spontaneous abortion and caffeine consumption ≥ 300 mg/day. In addition, a recent systematic review and meta-analysis by Greenwood *et al.* (2014) clearly demonstrated that each increment of 100 mg/day caffeine during pregnancy is associated with 14% increase in spontaneous abortion, 19% increase in stillbirth and 2% increase in preterm delivery. This finding is supported by an earlier study that showed a significant increase in risk of fetal demise in first and second trimester by consumption of 4-7 cups/day (95% CI, 1.08-1.63) and ≥ 8 cups/day (95% CI, 1.19-2.13) of coffee (Bech *et al.* 2005). Tolstrup *et al.* (2003) found that not only high caffeine consumption (>900 mg/day) during pregnancy but in pre-pregnancy period is associated with increased risk of miscarriage. Finally, Signorello *et al.* (2001) approached the link between miscarriage and caffeine consumption from a different angle and investigated N-acetylation status in women with CYP A1A2 genotype. They found a 3.17 fold increase in risk of miscarriage at 6-12 gestational weeks if any woman is a fast metabolizer and receives ≥300 mg/day caffeine. This result supports the above mentioned idea that single lower dose is more dangerous than higher dose in multiple aliquots. The exact mechanism of caffeine on miscarriage and other complications is not clearly elucidated in humans, but according to Kotsopoulos *et al.* (2009) caffeine intake was significantly associated with lower luteal total and free estradiol levels, but not luteal progesterone levels. Although, it is not always easy to apply human data to animals or vice versa, our results confirm adverse effects of high dose caffeine on reproduction (Minelli and Bellezza, 2011). Another important point is that the frequency of fetal

problems due to caffeine differs from one strain of rats to another with Osborne-Mendel and Sprague-Dawley strains being more sensitive than the Wistar strain (Nehlig and Debry, 1994).

5.4 Effect of caffeine on developing fetus

Another interesting finding, although it was not our primary goal, was that pups in the Cg had a significant growth retardation compared to pups in the Wg, with P0 weights of 5.79 ±0.54 g and 6.31 ±0.33 g, respectively, ($p < 0.0001$). There are many supportive studies showing that maternal caffeine consumption affects the fetus at any time throughout intrauterine life in humans (Jahanfar and Jaafar, 2015). Animal studies facilitated our understanding for fetal effect of caffeine. Fujii *et al*. (1972) found significant growth retardation in rat pups when mother received caffeine in diet that contains 0.25 or 0.50% caffeine by weight during whole and early pregnancy, but not in late pregnancy periods. In another study, Elmazar *et al.* (1982) supplied pregnant mice with 1.6 g/l caffeine in drinking water from days 5 to 18 of pregnancy. There was a significant weight difference between high dose Cg and controls. Another interesting study showed 120 mg/kg/day of caffeine intake from day E11 to end of pregnancy would affect fetal programming through hypothalamic-pituitary-adrenal axis not only in first generation but in the second generation pups as well (Luo *et al.,* 2014). All these findings indicate detrimental effect of caffeine on growing animal fetus and are very supportive for our results. Lower birth weight problem is also common in humans due to internal and external reasons (James, 2015). Globally, LBW has been estimated at 15.5% of all live born infants (Chen *et al.,* 2014). Interestingly, the question of whether caffeine has a risk for low birth weight in humans has still not been clearly answered. In 1985, an early systematic review showed tentative support that caffeine contributes to LBW (James and Paull, 1985). However, Brent *et al.* (2011) reported that moderate or even

higher amounts of caffeine consumption during pregnancy have no risk on developing fetus. A recent Cochrane meta-analysis did not confirm any significant benefit of moderate caffeine reduction on fetal weight compared to standard consumption (Jahanfar and Jaafar, 2013). The majority of studies have indicated 300 mg/day caffeine consumption as a threshold for LBW babies (Hoyt *et al.,* 2014).

Because of all these concerns and debates about possible associations with restricted fetal growth, birth defects, miscarriage and stillbirth in humans, several countries including the US and UK have applied precaution to limit caffeine intake to less than 200 mg/day immediately before and during pregnancy (Greenwood *et al.,* 2014; http://cot.food.gov.uk, 2008).

5.5 Caffeine exposure and A_1R, $A_{2A}R$ ontogeny

Although an objective of our study was to examine the effect of caffeine on post-natal expression and abundance of brain adenosine receptors, we observed there were other important findings that needed to be addressed.

Caffeine is a non-selective antagonist of A_1R and $A_{2A}R$ and when preterm infants are treated with caffeine for AOP, there is a blockage of A_3R as well (Aden, 2011). At plasma concentrations of 2 to 10 µg/ml, which are seen in humans after moderate to heavy intake of coffee, caffeine's effects are mostly mediated via blockade of adenosine A_1R and $A_{2A}Rs$ (Tofovic *et al.,* 2002). When preterm babies are treated with caffeine, higher plasma levels also inhibit A_3Rs and minimally block phosphodiesterases, $GABA_A$ receptors and release Ca^{2+} release from affected site (Aden, 2011).

The ontogeny of AR has been studied previously. Weaver (1996) reported a gradual increase in A_1Rs gene expression during fetal life of rats which was maximized on day E20 and

it was location specific. Pons and medulla were the top areas about having strong gene expression of A_1Rs. That could make possible A_1R detection in these areas on P0 but in the other brain regions. The same author has also shown that similar pattern for A_{2A}Rs gene expression which was maximum on day E20 (Weaver, 1993). Very interestingly caudate area has lost gene expression on P7 although it was detectable on P0. It suggests that AR gene expression and ontogeny is a dynamic process which could be affected from exposure to antagonists or perhaps agonists during fetal life.

A_1R expression could be detected as early as of E10 in pregnancy even when neural tissue first appears, but it is not fully functional until P14-21 in developing brain regions except medulla oblongata in rats (Weaver, 1996; Aden, 2011; Rivkees and Wendler, 2011). Similarly, A_{2A}R expression appears on E14, being later than A_1R, but again it is detectable on P14 in developing brain (Weaver, 1993). In our study we have detected increased A_1Rs in striatum but not detectable A_{2A}R on P0 by WB, so we also suggest the idea that adenosine receptor expression is dependent on type of the receptors and it could affect brain functionality that apparently shows difference between newborns and adults.

Caffeine and its effect on ontogeny of A_1R and A_{2A}R in developing brain has been studied by many authors. It was stated by Fredholm (1982) previously, chronic daily administration of caffeine at 60-75 mg/kg/day results in tolerance, which develops in 1-2 weeks and is maintained for up to two weeks after the cessation in humans. However, there is a debate about if the effect of caffeine on A_1R and A_{2A}R is through up or down regulation, or in changes in binding characteristics, gene expression or in receptor function (Holtzman *et al.,* 1991; Leon *et al.,* 2002). Intake of 0.8 g/l caffeine in drinking water for 7 days after birth has resulted in up-regulation in density of A_1R in cortex and neuroprotection against hypoxia in newborn pups

(Bona *et al.,* 1995). Ramkumar *et al.* (1988) found a significant increase in A_1R in adult rat brain cortex after 28 days of caffeine supplement in drinking water at 1 g/l. Similar results were reported by Fredholm (1982) and it was stated that such kind of up-regulation is found for several other types of receptors when an antagonist is given for a period of time. Caffeine showed also an up-regulatory effect on both A_1R and $A_{2A}R$ in different brain regions by postnatal administration at dose of 15 to 20 mg/kg/day from P2 to P6 to newborn rats (Gaytan *and* Pasaro, 2012). These results suggest caffeine up-regulates both A_1R and $A_{2A}R$.

In contrast, some authors found the effect of caffeine is only on some certain ARs and in some areas in the brain, like we found. Svenningsson *et al.* (1999) showed a down-regulation in $A_{2A}R$ in striatum but an increase in A_1R in lateral amygdala after 14 day of caffeine supply 0.3 g/l in drinking water in rats. Johansson *et al.* (1993) added caffeine 1 g/l to drinking water in rats for 12 days and they found a significant increase in A_1R but not $A_{2A}R$ in the brain. This is supported by another study which showed an up-regulation in A_1R but not in $A_{2A}R$ by chronic caffeine consumption (Riberio and Sebastiao, 2010). Our protocol provided 92 mg/kg/day caffeine citrate for pregnant rats. But, we found increased A_1Rs in the striatum by long term caffeine exposure, but not in the other areas. We also could not find any detectable $A_{2A}Rs$ in our study population. Therefore, we suggest that caffeine exposure during intrauterine period up-regulates A_1Rs only in some specific regions of the brain. This is supported by another study in which neonatal pups received caffeine at 20 mg/kg on postnatal day 2 and 15 mg/kg on days 3-6 and increased binding of A_1Rs in cortex, cerebellum and hippocampus but not in BS or hypothalamus (Guillet *and* Kellogg, 1991).

In another study, caffeine at a dose of 0.3 g/l in drinking water did not change A_1R ontogeny in cerebral and cerebellar cortex, hippocampus and thalamus in rats (Aden *et al.*,

2000). Since there was no change in mRNA expression of these receptors, the authors proposed that the effect of caffeine on these receptors is at the post-translational level. We found mRNA changes in specific brain areas. Our results showed a significant increase in gene expression of A_1R and $A_{2A}Rs$ in BS, but not in cerebral cortex or striatum by caffeine exposure during intrauterine period. BS is very important area for respiratory drive. First day of life in rats is equal to 24 weeks gestation in humans and P6 is equal to 32 weeks in newborn babies by brain development (Biran *et al.*, 2012). Also it is a well known fact that apnea, cessation of the breathing, occurs in early preterms rather than late infants in newborns. Seven percent of neonates born at 34 to 35 weeks gestation, 15% at 32 to 33 weeks, 54% at 30 to 31 weeks, and nearly all infants born at <29 weeks gestation or <1,000 g exhibit apnea (Zhao *et al.*, 2011). Although, this increase was no longer evident in P6 rats, our findings could help to understand if high dose of caffeine intake during the pregnancy could contribute to respiratory changes in preterms postnatally.

There are studies reporting opposite results to our study by using a similar protocol. Pregnant rats were treated with 1 g/l of caffeine during pregnancy, the same dose as in our study protocol, and an approximately 50% down-regulation in total A_1R but not in $A_{2A}R$ compared to the controls was observed (Leon *et al.*, 2002). Moreover, Lorenzo *et al.* (2010) found as much as 80% decrease in both A_1R receptor number and gene expression in mothers and their pups after exposure to 1 g/l of caffeine supplementation in drinking water during pregnancy and lactation periods. Kaplan *et al.* (1993) showed a relationship between caffeine dose and adenosine A_1R down-regulation: caffeine doses of 97 mg/kg/day (with mean plasma concentration of 2.7 µg/ml) and 194 mg/kg/day (with mean plasma concentration of 7.1 µg/ml) led to 20% and 69% decreases in A_1R in 6-8 weeks old mice. All these results indicate that the effect of caffeine

exposure on adenosine receptor abundance needs further studies since the results show discrepancy.

It should be noted however, plasma levels of caffeine were not the same even with the same dose of supplementation in the above mentioned studies. In our study, with the exception of A_1R in striatum, chronic caffeine administration in high dose did not increase or decrease either A_1R or $A_{2A}Rs$, detected by WB, in any brain region compared to saline. In addition, there was a protein-mRNA mismatch, as the increase in A_1R in striatum was not matched by an increase in rt-PCR product and the increase in rt-PCR product for A_1R and $A_{2A}R$ in BS was not linked to increases in receptor protein levels. The differences observed in P0 rats disappeared by P6. However, we conclude that changes in A_1R and $A_{2A}R$ ontogeny as we found should be studied further in animal models and its relevance to human respiratory behaviour should be observed in clinical studies especially in extreme preterm babies.

5.6 Relevance of our results to humans

According to Kotsopoulos *et al.* (2009) caffeine intake was significantly associated with lower luteal total and free estradiol levels, but not luteal progesterone levels. They concluded that caffeine may alter circulating levels of luteal estrogens and sex hormone binding globulin. Hahn *et al.* (2015) investigated whether there was any association between pre-conceptional caffeine intake and spontaneous abortion. There were no such associations except caffeine consumption ≥ 300 mg/day was associated with late spontaneous abortion. However, a recent systematic review and meta-analysis done by Greenwood *et al.* (2014) clearly demonstrated that each increment of 100 mg/day caffeine during pregnancy is associated with a 14% increase in spontaneous abortion, 19% increase in still birth and 2% increase in preterm delivery. Another large scale

study supported this finding. Compared to no coffee intake, there was a significant increase in risk of fetal demise in first and second trimester by consumption of 4-7 cups/day (95% CI, 1.08-1.63) and ≥ 8 cups/day (95% CI, 1.19-2.13) coffee (Bech *et al.,* 2005). A case-controled study also revealed a significant association between repeated (≥ 2) miscarriages and intake of coffee, tea, cocoa, chocolate and caffeine containing liquids by consumption of ≥300 mg caffeine per day. Interestingly this was not present in smokers (George *et al.,* 2006). Giannelli *et al.* (2003) found twice the risk of miscarriage with >300 mg/day caffeine intake during pregnancy compared to 151 mg/day caffeine. Tolstrup *et al.* (2003) found that not only high caffeine consumption (>900 mg/day) during pregnancy but in pre-pregnancy period is associated with increased risk of miscarriage. Another interesting study by Karyipidis *et al.* (2006) reported a significant association between first trimester miscarriage and caffeine consumption >500 mg/day in women who have CYP 1B1 432 Val/Val genotype. If any woman has this genotype, the risk of miscarriage is increased by 46% (OR: 1.46). The crude OR has increased to 7.42 and adjusted OR to 3.61 if this woman received caffeine >500 mg/day. Finally, Signorello *et al.* (2001) approached the miscarriage and caffeine relationship from a different angle and investigated N-acetylation status in women with CYP A1A2 genotype. They found that a 3.17 fold increase in risk for miscarriage at 6-12 gestational weeks if any woman is fast metabolizer and receives ≥300 mg/day caffeine. These last 2 studies can partially explain the diversity of adverse caffeine effect on women's reproduction. We used a higher dose of caffeine per kg than most studies, and we observed a very dramatic and detrimental effect on pups by size and numbers. However, there might be some differences between human and animal study results for caffeine. First, the critical cut-off for detrimental dose of caffeine could be different between humans and rats. Second, animal experiments usually test high doses of caffeine and it is

difficult to compare such high doses in pregnant women. Therefore, the effect of caffeine on reproduction should be investigated more precisely.

6. Conclusion:

Based on our results, we conclude that antenatal high dose of caffeine exposure has adverse effects on animal productivity, intrauterine growth and postnatal weight gain. In addition, antenatal caffeine use would not change caffeine metabolism in newborn rats loaded with caffeine postnatally. Our study has also shown that when it is consumed chronically and at high dose during pregnancy, maternal caffeine crosses placenta and provides a satisfactory caffeine level in their offspring on P0. However, this exposure altered ARs in specific areas of the barin. An upregulated A_1Rs in the striatum was detected by WB. Moreover, BS A_1R and A_{2A}R gene expression were both upregulated in Cg. This upregulation has disappeared by P6. There was no remarkable effect of caffeine exposure on AR ontogeny by P6. These results tell us that high dose of caffeine exposure during intrauterine period can modify adenosine receptors, especially in the BS and striatum. However, results of this modification need to be supported by an *in vivo* respiratory test which is the subject of the next chapter.

Chapter V:

Effect of caffeine intake during pregnancy on ventilation under hypoxic challenge in newborn rats

1. Background:

1.1 Characteristics of respiratory physiology in newborns

The development of respiratory control starts early in gestation but continues to mature for weeks or months after term birth (Neumann *et al.,* 2014).The breathing pattern of preterm and term infants is often irregular due to immaturity of central control system at brainstem which is guided by peripheral and central chemoreceptor responses (Neumann *et al.,* 2014; Zhao *et al.,* 2011). Therefore, nearly all premature infants born at < 29 weeks or 1000 g and 85% born at < 34 weeks exhibit AOP (Schmidt *et al.,* 2007).

Apnea is defined as a pause of breathing for more than 15-20 sec, accompanied by oxygen desaturation ($SpO_2 \leq 80\%$ for ≥ 4 seconds) and bradycardia (heart rate < 2/3 of baseline for ≥ 4 sec), in infants born less than 37 weeks of gestation (Zhao *et al.,* 2000). In mature brain, hypercapnia causes an increase in tidal volume and respiratory rate, but this response is very weak in early preterm infants (Neumann *et al.,* 2014). Therefore, it results in recurrent episodes of apnea and chronic hypoxia that all are harmful for a developing brain (Tye *et al.,* 1993) and also causes dysfunction of the gut or other organs (Henderson-Smart and De Paoli, 2010). Moreover, these infants have a smaller oxygen reserve, higher risk for functional residual capacity loss, increased airway resistance due to smaller airway size and impaired ventilatory response to hypercapnia and hypoxia (Neumann *et al.,* 2014). The methylxanthines such as aminophylline, theophylline, and caffeine are common medications that have been used for

improving respiratory physiology in newborns (Aranda *et al.,* 2010). Caffeine and aminophylline are also used for treatment of AOP (Schmidt *et al.,* 2007). If medications do not provide sufficient effect, then other strategies such as prone positioning, nasal intermittent positive pressure ventilation or continuous positive airway pressure may be used (Zhao *et al.,* 2000).

1.2 Effect of caffeine and other methylxanthines on respiratory physiology

Establishment of a normal respiratory pattern is a major developmental milestone for many premature infants, and therefore caffeine is currently a worldwide therapy (Zhao *et al.,* 2000; Schmidt *et al.,* 2007; Henderson-Smart and De Paoli, 2010). Caffeine effect is attributed to its action on the CNS where it stimulates min ventilation and improves sensitivity and /or responsiveness to changes in arterial O_2 and CO_2 levels (Montandon *et al.,* 2008; Yang *et al.,* 2010). Caffeine is a weak bronchodilator compared to theophylline, a dimethylxanthine (Aranda *et al.,* 2010). Therefore, it decreases airway resistance and increases airway compliance in normal conditions. Caffeine exerts its effects by inactivating AR by competitive inhibition (Comer *et al.,* 2001). Both A_1R and $A_{2A}R$ are main targets for caffeine (Parkinson *et al.,* 2000). Adenosine A_1Rs, are located both pre- and post-synaptically throughout the brain, including cerebral cortex, striatum, hippocampus and cerebellum (Fredholm *et al.,* 2005; Dunwiddie and Masino, 2001; von Lubitz, 1999). Adenosine $A_{2A}Rs$ are highly expressed in striatum, nucleus accumbens and olfactory tubercle where they co-localize with dopamine D2 receptors (Stone and Ceruti, 2009). Respiratory stimulant effects of caffeine are mainly by antagonism of brainstem A_1R and $A_{2A}R$ located on groups of neurons involved in the generation of respiratory rhythm (Montandon *et al.,* 2008; Yang *et al.,* 2010). However, chronic and acute intake of caffeine may affect AR in different and even opposite ways (Ribeiro and Sebastio, 2010).

2. Rationale

Consuming caffeinated beverages is very common habit worldwide and most pregnant women continue to drink caffeinated beverages during pregnancy in developed countries (Aden, 2011; Yang *et al.,* 2010). Average caffeine intake is estimated to be 164 mg/day among women 18-34 years and 125 mg/day among pregnant women although it is quite variable among individuals (Browne, 2006). Caffeine almost totally passes through the placenta and blood-brain barrier (Aden, 2011). Its metabolism is impaired in pregnancy and the main metabolizing enzyme, CYP 1A2 is absent in fetuses and neonates, leading to accumulation in fetal tissues (Aden, 2011; Chen *et al.,* 2015, Okubo *et al.,* 2015). High caffeine intake during pregnancy is a risk factor for withdrawal, which presents with vomiting, intermittent bradycardia, periodic breathing, apnea, tachypnea and jitteriness during the first days of life (McGowan *et al.,* 1988).

On the other hand, apnea which is a cessation of breathing, is treated with methylxanthines in newborn babies and caffeine, the most popular one, has also been found to be neuroprotective in this population (Schmidt *et al.,* 2007). In normal conditions, caffeine increases mean respiratory rate and min volume, stimulates central respiratory centres, increases pulmonary blood flow and increases the sensitivity of central medullary areas to hypercapnia. In premature neonates, caffeine was shown to improve the compliance of the respiratory system, reducing the strength of the Hering Breuer reflex and the inspired oxygen requirements over the 7-day study period (Comer *et al.,* 2001; Ribeiro *et al.,* 2010). In almost every corner of the world, caffeine has been used as a respiratory stimulant in neonatal intensive care units (Schmidt *et al.,* 2007).

However, there is a gap between the facts that maternal caffeine intake during pregnancy varies among the mothers, and caffeine response in newborn babies shows variations and some

babies need extra doses of caffeine for better clinical response. In our previous study, chronic caffeine consumption during pregnancy was associated with upregulation of A_1Rs in the striatum at P0 and upregulated gene expression of both A_1R and A_{2A}Rs in the BS at P0. It is well known that BS maturation might affect the ventilatory response in animals and human under physiological conditions and hypoxemia. This is more evident in preterm babies and caffeine has been used to treat this condition. Therefore, in the present study, we tested for a connection between intrauterine caffeine exposure and postnatal respiratory response to caffeine in a respiratory physiology study using hypoxic newborn rats.

3. Objective/Hypothesis

Our hypothesis is that caffeine will act as a respiratory stimulant in rat pups exposed to hypoxia but that prenatal caffeine exposure will modify this response.

4. Methodology

Pregnant rats were kept in a ventilated room under controlled conditions of lighting and optimal temperature (21°C, 12:12-h dark-light cycle, with lights on at 08:00 and off at 20:00) with free access to food and drinking water (caffeinated or not) in Animal Care Facility of the University of Manitoba. The University of Manitoba Animal Care Committee approval for the experimental procedures was obtained, and all protocols were performed in accordance with the guidelines detailed by the Canadian Council on Animal Care. When the pregnancy was confirmed by animal care technician by detection of vaginal plug, the first step of the experiment

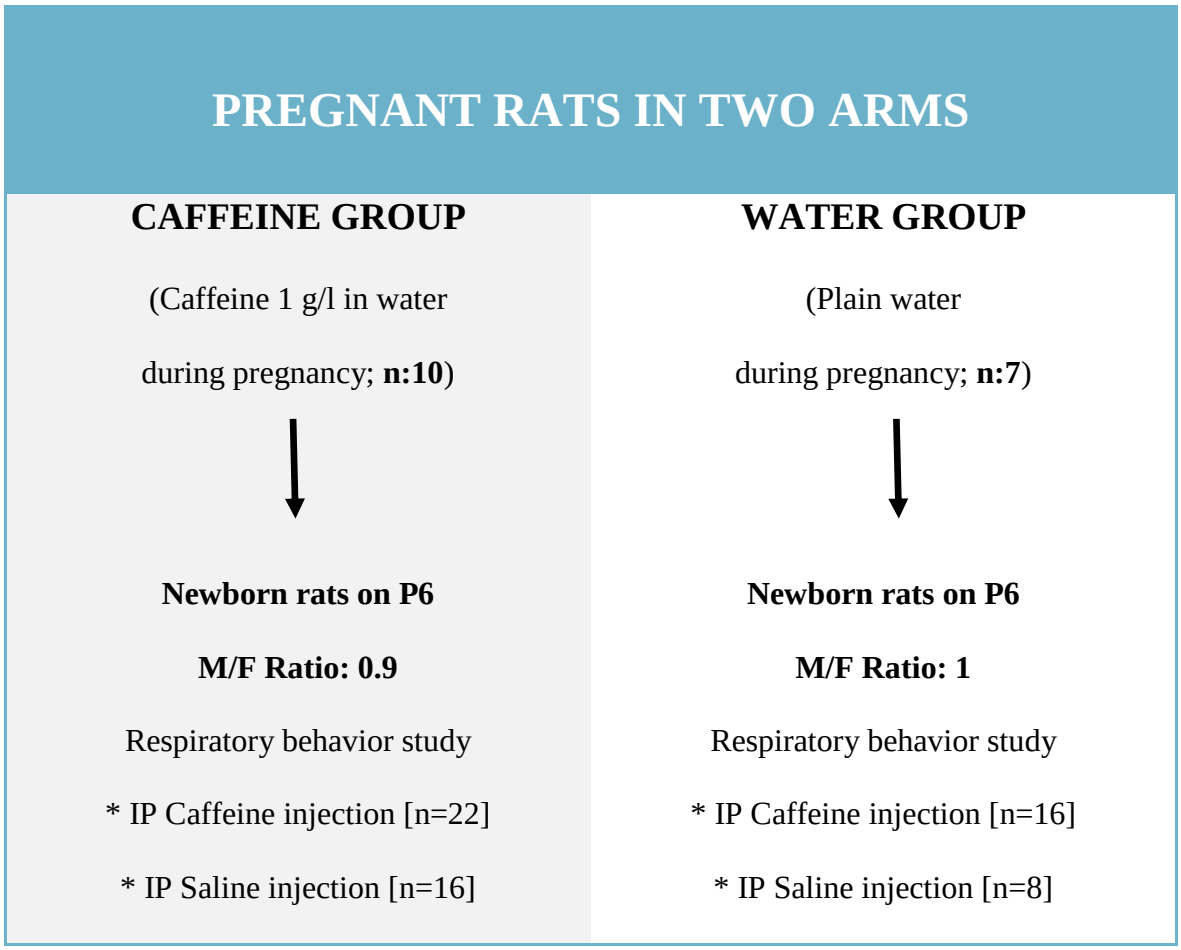


Figure 1: Study design is summarized on the flow chart

was initiated. The rats were stratified into 2 groups. First group (aka, Cg) received 1 g/l of caffeine citrate with drinking water from gestation day 1 onwards during the entire gestation period. Second group of rats (aka, Wg) were control animals and received caffeine-free tap water (Figure 1). Caffeine and water bottles were refreshed with new ones every other day. The animals were weighed at the same time. At the day of delivery caffeine supplementation was terminated and the animals were provided with plain drinking water. Each study group received the same care otherwise.

The pups were kept with their mothers receiving normal maternal care until P6. On P6 groups were subdivided into 4 subgroups. From the Cg, the first subgroup received caffeine citrate (at 60 mg/kg/dose IP) and the second subgroup received an equal volume of IP saline based on their body weights. From the Wg, the third subgroup received caffeine citrate (at 60 mg/kg/dose IP) and the fourth subgroup received an equal volume of IP saline based on their body weights (Figure 1).

4.1 Respiratory behavioral study in P6 pups under hypoxic stimuli

All subgroups were used in a respiratory behavioral study for 110 min starting 10 min after the injection.

For respiratory behavior test, Buxco whole body unrestrained flow-through plethysmography was used. Data were recorded by the software Buxco XA Version 2.7.9 (Buxco Electronic Inc. USA). The software recorded signals simultaneously during the experiment from 4 chambers designed for small rodents. Signal calibration was performed daily. The protocol for wave recording was adjusted according to Drarbaugh & Fern method in the software. In addition respiratory waves were recorded from the screen through a data acquisition software from beginning until the end of each experiment. The temperature inside the plethysmograph chambers was fixed at 26°C using a heated pad system. Rectal temperature was measured before and at the end of the experiment using a thermocouple for small rodents. The gas flow through the plethysmograph was set at 200 ml/min, constantly measured with a mass flowmeter. Oxygen and nitrogen cylinders were connected to ProOx 110 Oxygen Controller (Biospherix, NY, USA). Tidal volume was corrected according to barometric pressure and 26°C air temperature. The following parameters were recorded:

a-) Respiratory frequency as breaths per min; b-) Tidal volume as ml/kg body weight; c-) Corrected min volume (min volume= TV*Frequency of breathing in 1 min) was calculated and it was corrected later according to animals weight and was reported as ml/kg/min; d-) Inspiratory/expiratory ratio by calculation of inspiratory time divided to expiratory time and was reported as percentage; e-) Tidal mid expiratory flow (EF50; in ml/sec) was calculated as the tidal flow at the midpoint (50%) of expiratory tidal volume and was an index of bronchoconstriction.

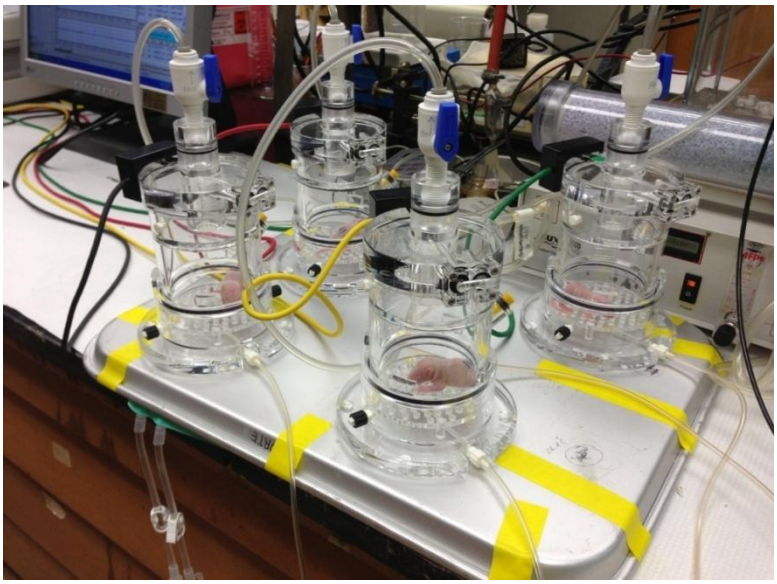


Figure 2: A photograph from the experiment which shows unrestrained rat pups in the plethysmograph chambers.

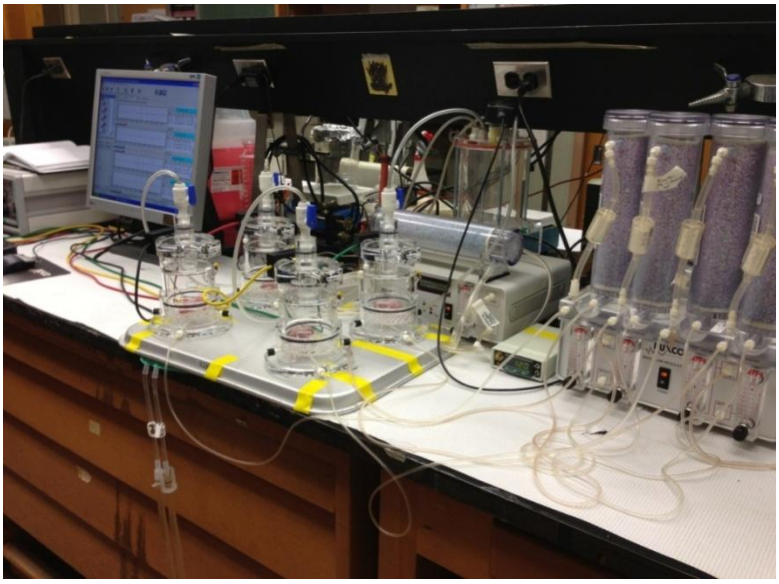


Figure 3: Another photograph shows most of the equipment of the experiment such as flowmetre, air pump, air purifier and oxygen controller. Chamber volume changes, representing breathing activity, were recorded and analyzed by computer.

As a first step, the pups were weighed and then received IP injection of either 60 mg/kg caffeine or isovolumetric saline. Then they were placed in plethysmograph chambers and the recording was initiated at 10 min post injection. After 20 min of acclimation period, a hypoxic challenge was initiated by exposing pups to 12% O_2 for 20 min with a 10 min recovery period. Finally the pups were kept in the chambers at rest for an additional 60 min which was divided into 20 min periods. The total duration of the experiment including the 10 min post-injection time was 120 min. After the experiment, body temperature was checked for each pup.

4.2 Statistical analysis

Continuous variables were expressed as mean ± SD. Statistical analyses were performed using SPSS software program (SPSS Version 20, USA).

Each of the outcome measures (Frequency, corrected tidal volume (CTV), corrected minute volume (CMV), I/E, EF50) in the groups were analyzed using a repeated measures analysis of variance (ANOVA) with treatment group as between group factor and repeated measure times as within group factor. All corrections were done according to rat`s weight, since absolute numbers were different from one animal to other one, but weight was important factor affecting those numbers. If there was any significant difference among four groups (Caffeine-Caffeine (CC), Caffeine-Saline (CS), Water-Caffeine (WC) and Water-Saline (WS)) then Tukey post-hoc test was performed.

In addition, over time changes in respiratory outcomes were compared to baseline and the difference was calculated as percentile of positive or negative changes by the time on time axis with the same method as described above. All p-values were two-sided, and significance was set at a value of 0.05. Data were graphed using Graphpad Prism software program (Version 5.04, USA).

5. Results

5.1 Comparison of mean values in respiratory function tests among the groups

Overall, no effect of prenatal caffeine treatment on respiratory function before, during or after an hypoxic challenge was observed in P6 rats. The only difference was on RR and groups that received caffeine showed a significant decrease in RR which was compensated by increased TV. There was also a trend for an acute post-natal caffeine treatment to increase TV, MV and

EF50 during hypoxia and re-oxygenation was noted (Figures 4A, 5A and 7A). However, there were no significant differences among the groups in CTV, CMV, I/E and EF50 ($p>0.05$) (Figures 4-7). After correcting for baseline responses, a trend for an acute postnatal caffeine treatment to decrease CMV and EF50 was noted but these differences were not statistically significant (Figures 5 and 7; lower panels). Similarly, there were no statistical differences among the groups in CTV or I/E ratios (Figures 4, 6; lower panels).

IP injection of caffeine showed a significant effect on RR on neonatal pups regardless their mother has received caffeine during the pregnancy (Figure 8). This was more evident before hypoxia period initiated. The CC treatment groups showed significantly greater suppression of RR than CS and WS groups ($p<0.05$). By increased TV it was very well compensated, thus we were able to see higher MV at the end, but the difference in VT and MV were not statistically significant, in CC and WC groups compared to others. It is also very interesting to see that the groups received caffeine started the experiment with better TV probably by stimulation of respiratory force. At the end of experiment, MV was very similar in all groups and initial difference was not evident. In sum, the results indicate that caffeine's effect is mainly on respiratory strength.

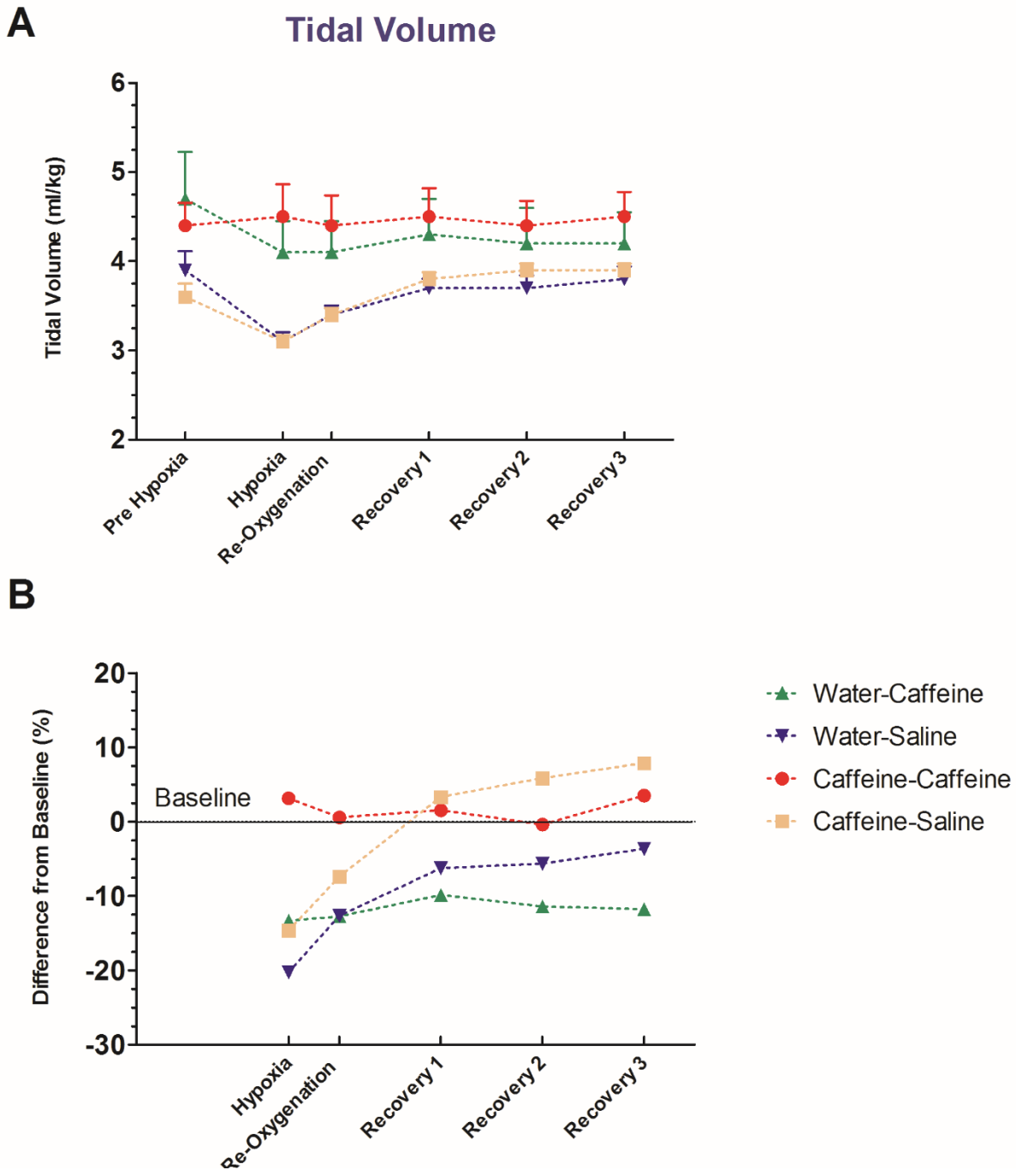

A
Tidal Volume
Tidal Volume (ml/kg)
6
5
4
3
2
Pre Hypoxia
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
B
Difference from Baseline (%)
20
10
0
-10
-20
-30
Baseline
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
Water-Caffeine
Water-Saline
Caffeine-Caffeine
Caffeine-Saline

Figure 4: Mean of actual TV over study period and ratio of the difference between each period from baseline.

A. Mean values of TV over study period did not show any significant difference between the groups. However, hypoxia caused a depression in TV briefly in most groups. Note the effect of caffeine injection on protecting TV although a statistical significance was not achieved (mean ± SE).

B. Corrected TV showed a decrease during hypoxia p(ecrea)-2(se)9( )-3(d)5(u)-4(rin)-5(g)11( )-3

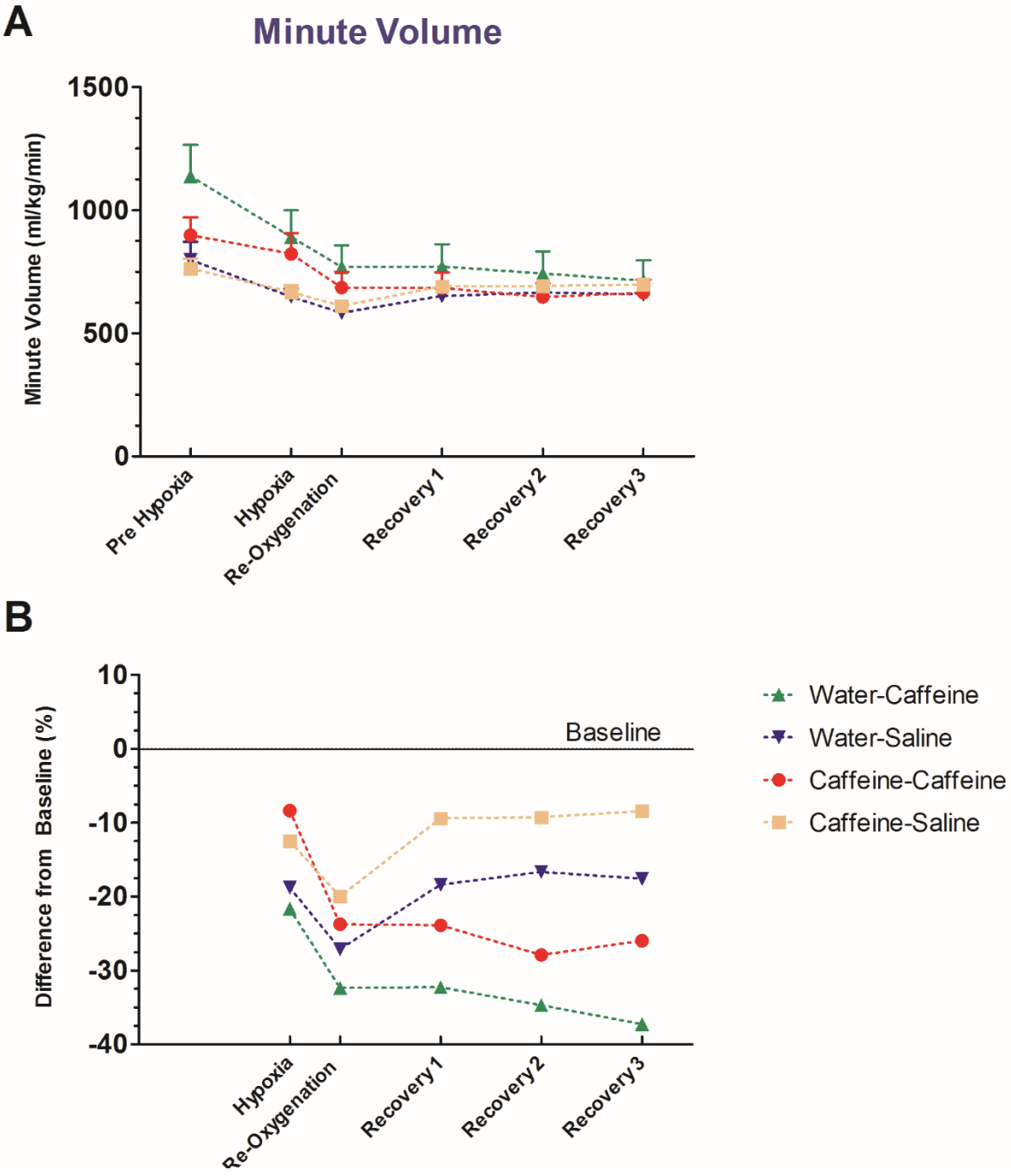
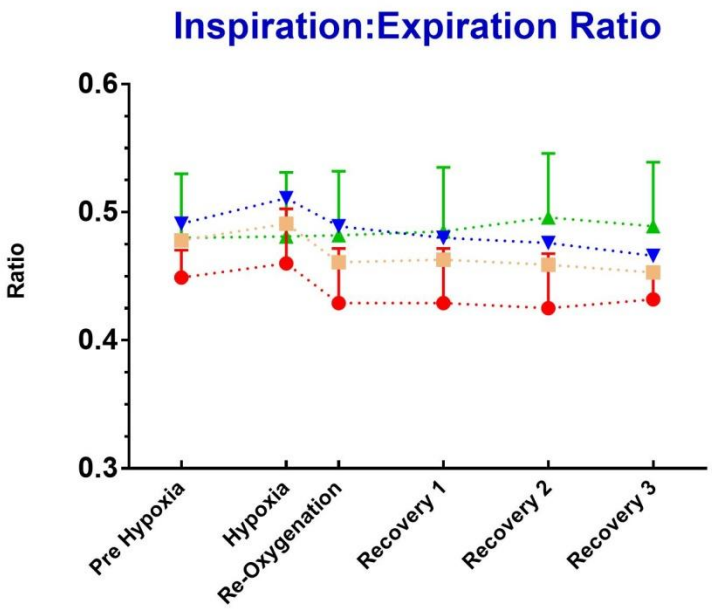

A
Minute Volume
Minute Volume (ml/kg/min)
1500
1000
500
0
Pre Hypoxia
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
B
Difference from Baseline (%)
10
0
-10
-20
-30
-40
Baseline
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
Water-Caffeine
Water-Saline
Caffeine-Caffeine
Caffeine-Saline

Figure 5: Mean of actual MV over study period and ratio of the difference between each period from baseline.

A. Mean values of MV over the time showed a depression after hypoxia event. There was a modest recovery after that in most groups (mean ± SE).

B. Corrected MV showed a sharp decrease in all groups during hypoxia and reoxygenation periods. Recovery towards baseline was observed in CS and WS groups. Overall, the results were not statistically significant among the study groups ($p>0.05$).

A


Inspiration:Expiration Ratio
Ratio
0.6
0.5
0.4
0.3
Pre Hypoxia
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3

B

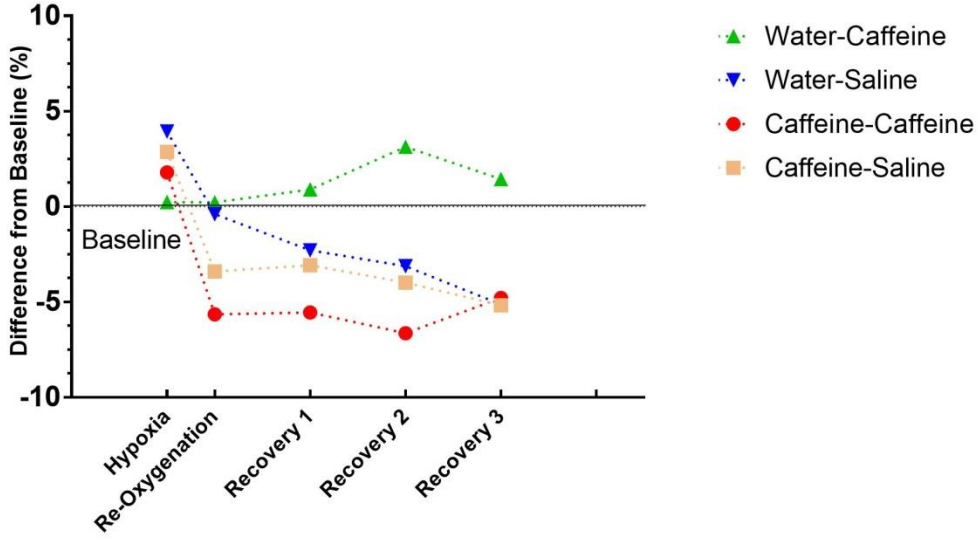

Difference from Baseline (%)
10
5
0
-5
-10
Baseline
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
Water-Caffeine
Water-Saline
Caffeine-Caffeine
Caffeine-Saline

Figure 6: Mean of actual I/E ratio (inspiration time/expiration time in a single breathing cycle) over study period and ratio of the difference between each period from baseline.

A. Mean values of I/E ratio showed an increased inspiratory component in most groups during hypoxia period.

B. A time graphic demonstrated fluctuation relative to baseline during hypoxia and reoxygenation periods in I/E ratio. There was longer inspiratory time during hypoxia which was reversed during reoxygenation and remained below baseline in all groups except WC. However, these results are not significant among the groups ($p>0.05$).

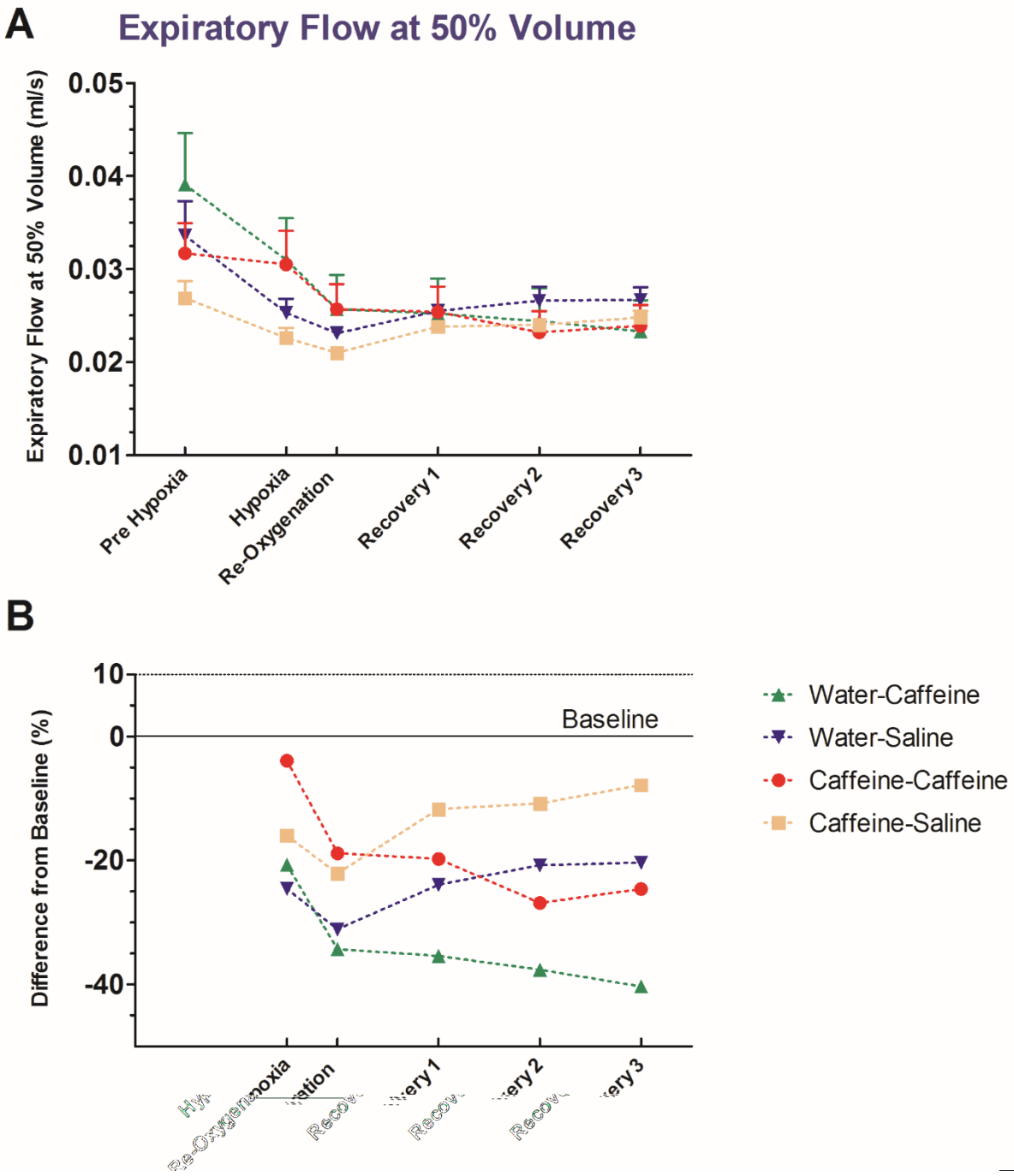

A
Expiratory Flow at 50% Volume
Expiratory Flow at 50% Volume (ml/s)
0.05
0.04
0.03
0.02
0.01
Pre Hypoxia
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
B
Difference from Baseline (%)
10
0
-20
-40
Baseline
Water-Caffeine
Water-Saline
Caffeine-Caffeine
Caffeine-Saline

Figure 7: Mean of actual numbers over study period and ratio of the difference between each period from baseline for Expiratory Flow 50 (EF50).

A. Mean values of EF50 showed a decreased expiratory mid flow during hypoxia and reoxygenation. It indicates bronchial resistance is increased in all group similarly (mean ± SE).

B. Expiratory flow at 50%, known as expiratory flow at 50% of expiratory tidal volume, is an indicative marker of bronchial resistance and it is shown as ml/sec. It showed a sharp decrease indicating increased bronchoconstruction during hypoxia period. The increased bronchoconstriction partially improved during the recovery periods in CS and WS groups. The differences among the treatment groups were not statistically significant ($p>0.05$).

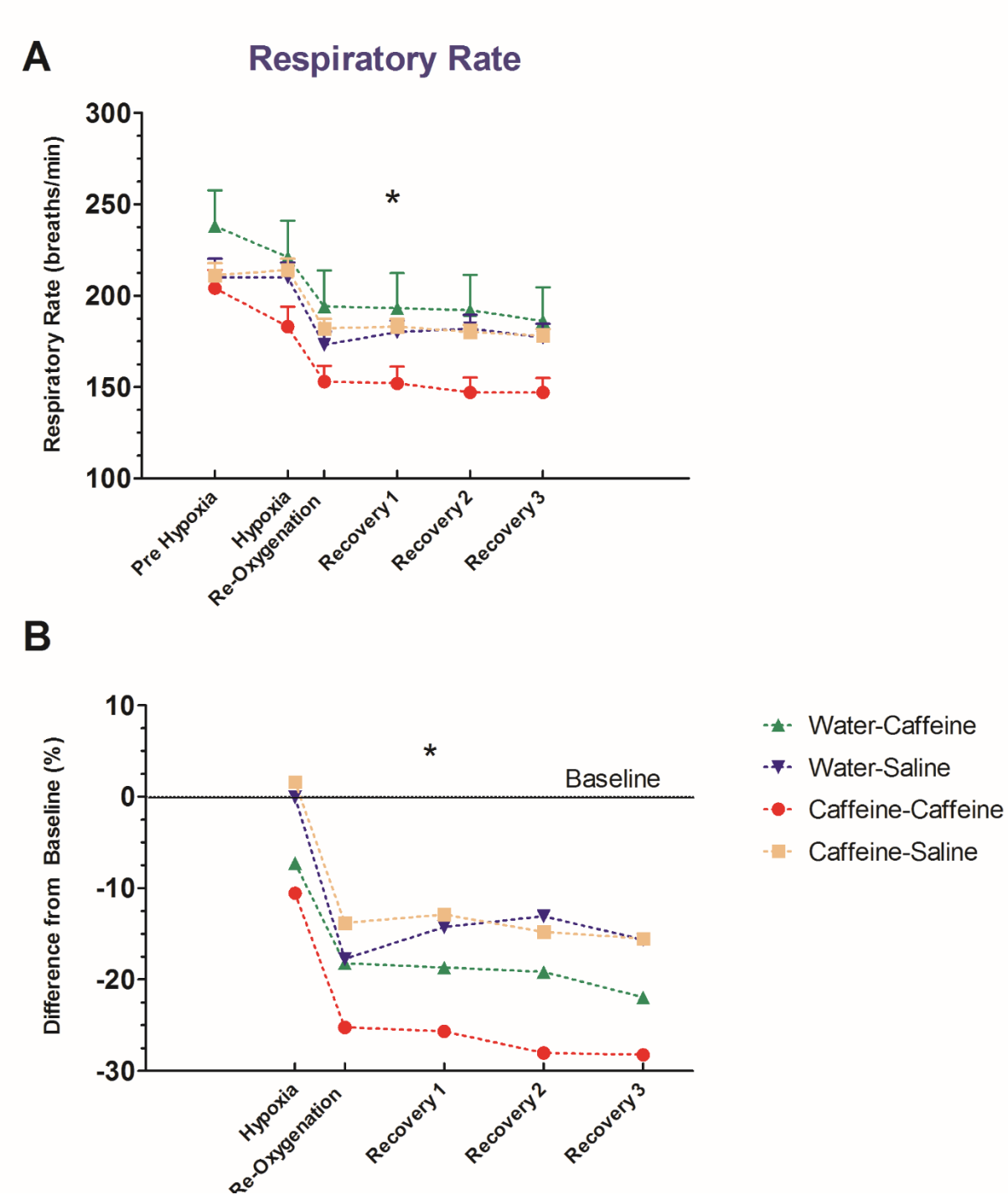

A
Respiratory Rate
Respiratory Rate (breaths/min)
300
250
200
150
100
*
Pre Hypoxia
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
B
Difference from Baseline (%)
10
0
-10
-20
-30
*
Baseline
Hypoxia
Re-Oxygenation
Recovery 1
Recovery 2
Recovery 3
Water-Caffeine
Water-Saline
Caffeine-Caffeine
Caffeine-Saline

Figure 8: Mean of actual numbers over study period and ratio of the difference between each period from baseline for respiratory rate.

A. Mean values of respiratory rate tests showed a significant difference among 4 groups on time intervals (*p<0.01). CC group showed a significant difference from CS (p<0.05) and WC (p<0.001) groups. No other significant differences between groups were evident (mean ± SE).

B. Differences in respiratory rate for specific time intervals compared to baseline levels. Again, there was a significant difference among the groups (*p<0.01). CC group showed a significant difference in comparison to CS (p<0.01) and WS (p<0.05) groups. There were no significant differences among other groups.

6. Discussion

6.1 Caffeine effect on lung physiology during hypoxia/reoxygenation

Maternal caffeine consumption may have long-term consequences and effects on respiratory control systems in their offspring (Cayetanot *et al.,* 2009). We attempted to characterize this effect using a sustained hypoxic challenge in newborn rats. When a moderate hypoxia is sustained for 5-30 min in adult animals this is considered as prolonged hypoxic exposure (Powell *et al.,* 1998). Our expectation was to provoke a hypoxic ventilatory response, which consists of a biphasic respiratory course characterized by a stimulation followed by a depression in these newborn animals (Pamenter *et al.,* 2015, Koos *et al.,* 2004, Zhao *et al.,* 2011,

Picard *et al.*, 2008). Similar to sustained hypoxia, brief intermittent hypoxia also results in a biphasic response in which an increase in MV, RR and TV is followed by a decrease in all these parameters in early newborn rats. This is followed by a compensatory increase in TV (Julien *et al.*, 2008). Our results showed a typical depression in ventilatory response to hypoxia. Only exception was CC group and showed a compensatory increase in their TV by exposure to hypoxia. However during recovery period all pups showed an effort to correct their respiratory response by increasing TV and stabilizing their RR. This is well characterized phenomenen. In adult animals, however the course is different. Acute intermittent hypoxia induces a gradual increase of min ventilation, called long-term facilitation (Julien *et al.*, 2008). This is not well observed in preterm animals or humans (Koos *et al.*, 2004). With prolonged hypoxia, Powell *et al.* (1998) found a "roll off" or hypoxic ventilatory decline (HVD), a decrease in ventilation for up to 60 min after normoxia (Powell *et al.* 1998). Some authors believe this depression is a protective metabolic response that decreases oxygen requirement (Pamenter *et al.*, 2015). In our study, it was evident that sustained hypoxia caused respiratory depression mainly in RR and MV in all groups. In this period TV was the main protective mechanism against this depression and showed a compensatory increase over time. It was more obvious in CC group but the results did not show statistical significance. Julien *et al.* (2010) reported that caffeine injection can increase min ventilation under normoxemia but not under brief or sustained hypoxia. Indeed, caffeine group (both CC and WC groups) entered to study with higher MV compared to other two groups. This difference diasappeared over the experimental period. It can imply that clinical effect of caffeine can show variations based on newborn's respiratory distress, which can mask the effect.

Our next question was to elucidate prenatal caffeine exposure on hypoxic ventilatory response (HVR). In the literature, it was shown that newborn rats exposed to caffeine during

intrauterine period and no post-natal supplementation of caffeine through mothers` milk, exhibit respiratory responses during hypoxia that are more depressed and may even result in apnea (Bodineau *et al.,* 2006). But a most concerning report about effect of maternal caffeine intake on newborns was from humans and it was shown that caffeine intake more than 400 mg/day or more is associated with fetal harm and sudden infant death syndrome which is the most severe form of hypoxic event and respiratory depression (Ford *et al.,* 1998). Therefore, we aimed to understand this is a reproducable situation and if there is any association with ARs in the last two studies. Apnea was another parameter of interest but the results were too subjective due to technical difficulties and it was not included to the results. Picard *et al.* (2008) demonstrated that maternal caffeine exposure during gestation (0.2 g/l in drinking water) and lactation attenuates HVR. However, this study did not include any postnatal caffeine loading as is used to be a routine procedure in preterm human newborns. However, Bodineau *et al.* (2006) found that if any newborn rat receives prenatal caffeine through placenta and postnatal caffeine through lactation, the severity of respiratory depression is less. There was another earlier study from the same author who investigated maternal caffeine intake on respiratory drive in hypoxia and normoxia by electrophysiology (Bodineau *et al.,* 2003). The results showed maternal intake of caffeine at 0.2 g/l increases RR compared to controls on P1-3 pups *in vitro* normoxemia. However, there was a significantly depressed RR in brain stem samples exposed to maternal caffeine under hypoxic stimuli. This result is very supportive for our observation. There was a significantly higher respiratory depression in our CC group compared to others. It could show maternal intake of caffeine has some effect on respiratory drive under physiological and pathological conditions. Unfortunately there is no clear answer so far from clinical studies. There is one study that showed a similar finding in newborn humans that received excessive amount of caffeine during

gestation and the babies showed apnea due to caffeine withdrawal on first days of life (Hentges *et al.,* 2010). We could not count apneas in our study due to excessive artefacts on respiratory waves due to movements of rat pups in plethysmograph chambers, but we could conclude that there was a significantly higher respiratory depression in CC groups compared to others by moderate hypoxia. Our explanation why we could not see this depressive effect in other parameters are a-) immaturity of the respiratory center in brain and periphery (Pamenter *et al.,* 2015) and/or b-) different hypoxia protocols (i.e., brief vs. prolonged, intermittent vs. sustained) would modify the efficacy and protective effect of caffeine and would give different results in ventilatory responses (Powell *et al.,* 1998). Unfortunately, current literature does not provide sufficient data about hypoxic stimuli and ventilatory responses in newborn rats and humans. Only a few studies have been concerned with the developmental consequences of caffeine exposure on breathing (Picard *et al.,* 2008). We hope our study could add some information on this issue. We chose to use sustained hypoxia since brief intermittent hypoxia caused a wide fluctuation in oxygen content among the chambers and we were concerned that the inconsistencies in oxygen levels would affect the results. With prolonged hypoxia, Powell *et al.* (1998) found a "roll off" or HVD, a decrease in ventilation for up to 60 min after normoxia, which was also very evident in min ventilation in our study during the recovery period but the recovery was consistent in all groups.

Respiratory depression, a major reason for hypoxia in newborn babies, is considered dangerous and therefore methylxanthines especially caffeine have been used effectively for several decades as a respiratory stimulant (Montandon *et al.,* 2008, Mosca *et al.,* 2014). Caffeine perhaps by decreasing hypoxia numbers and duration, improves neurodevelopmental outcome (Schmidt *et al.,* 2007). Caffeine increases ventilatory drive, acts directly on the respiratory

control center in the brainstem and improves sensitivity to any change in arterial O_2 content (Julien *et al.,* 2010). The exact mechanism of action in respiratory depression remains poorly defined (Bhatt-Mehta and Schumacher, 2003). It is thought that caffeine effects are mainly by antagonising AR as adenosine is known to be a central respiratory depressant and a peripheral respiratory stimulant (Bhatt-Mehta and Schumacher, 2003). The role of adenosine on respiration has been shown by A_1R activation, which results in a significant decrease in respiratory frequency in BS and upper spinal cord (Gaytan *et al.,* 2006). In other words, centrally applied adenosine analogs depress respiratory rate and depth via A_1R in the BS (Herlenius *et al.,* 2002). AR were increased by high doses of caffeine consumption during pregnancy in the pontine area, but this resulted in depression of respiratory centers in the medulla oblongata (Herlenius *et al.,* 2002). That indicates antenatal caffeine use could be detrimental for postnatal respiratory drive. Moreover, our results did not show any significant effect of prenatal caffeine exposure on AR ontogeny and respiratory drive. Although caffeine is a well-known respiratory stimulant, it had no protective effect on ventilation when newborn pups were exposed to hypoxia. Similarly, Montandon *et al.* (2008) reported that the adenosine A_1R antagonist DCPCX did not make any change in ventilation in 20 days old rats. In another study, antenatal low dose (0.3 g/l) caffeine intake did not affect number or distribution of A_1R or mRNA, but inhibited more BS respiratory rhythm (Herlenius *et al.,* 2001). Ventilatory responses to hypoxia in vertebrates are not the product of a single mechanism, but are a complex interplay between several distinct mechanisms as stated by Powell *et al.* (1998). Mosca *et al.* (2014) concluded that methylxanthine therapy is likely mediated via inhibition of cAMP-dependent phosphodiesterase. Therefore, in the future it might be worthwhile to look at other caffeine related mechanisms such as dopaminergic,

serotoninergic and GABAergic receptors and the effect of caffeine on these receptors during hypoxia.

7. Limitations

We used whole body plethysmograph technique in our study to measure ventilatory parameters. At this stage, it has a capacity to measure TV, RR, MV and other ventilation associated parameters effectively. As this is an unrestrained and non-sedated animal model, it might have errors during the assessment due to movement and anxiety artefacts. However, these artefacts would be similar for all study subjects and not specific to any study subgroup. Another important factor that was stated by Julien *et al.* (2010) is the effect of sleep-wake states on the experiment. These authors reported that these cycles are very brief, therefore we believe they would not affect the results in our experiment. As another limitation, apnea was very important target for us to measure, but there was too much artifact due to body movements when we counted apnea manually. It needs more sophisticated and computerized system for better and easier counting of apnea.

8. Conclusion

Based on our results, we conclude that respiratory responses in P6 rat pups after long-sustained moderate hypoxia has been affected if newborns were exposed to both prenatal and postnatal caffeine exposure. There is a respiratory depression in these pups after hypoxic event as expected. Rats in the CC group showed significantly decreased RR, but antenatal and postanatal caffeine could improve TV response and help pups achieve better compensation. Therefore, intrauterine caffeine exposure could be a contributing factor to postnatal respiratory

variability in response to caffeine treatment. However, other potential mechanisms such as genetic polymorphisms affecting caffeine metabolism should also be considered for these differences. Our previous results did not indicate any ontological changes in ARs by long-term caffeine exposure. Apnea and sudden infant death are big concerns in newborns. Therefore, further efforts for better understanding the relationship between caffeine and respiratory drive with ARs are essential and could be even bettter if other receptor mechanisms such as $GABA_A$ and their relationships with adenosine receptors is addressed.

Chapter VI:

General Discussion

Adenosine and its receptors are very functional and play roles in uncountable physiological and pathological health conditions (Bodenmann *et al.,* 2012, Borea *et al.,* 2016; Huang *et al.,* 2014, Ribeiro and Sebastiao, 2010; Shinohara *et al.,* 2013; Stone *et al.,* 2009). In this thesis, we looked at this modification from intrauterine period and extended to postnatal life. Therefore, in our studies we have focused on both modification of ontogenical development and function of ARs by using non-selective antagonist, caffeine, before and after birth. Pregnancy and early postnatal life was selected as a target time frame because of our intention of extending the results from bench to bed in clinical settings in the future. Modification of adenosine release or receptors by agonists and antagonists or its transporters was also another target in this thesis and we believe modification of adenosine levels would help for developing new drugs targeting many neurological conditions. Modification of adenosine transporter function, especially ENT1, has received increased attention in many recent research studies (Nguyen *et al.,* 2015; Cui *et al.,* 2013; Parkinson *et al.,* 2009). Such modification will affect adenosine influx or efflux and thereby intracellular or extracellular adenosine concentrations (Pedata *et al.,* 2015, Parkinson *et al.,* 2005; Xu *et al.,* 2015). In our studies, we have used a genetically altered mouse model, which over express ENT1 in neurons and have the potential for faster influx/efflux of adenosine through cell membrane. Our *in vivo* results in this thesis were consistent with our previous *in vivo* and *in vitro* results (Parkinson *et al.,* 2009; Kost *et al.,* 2011). Together with current results, we strongly believe that adenosine source is mostly extracellular during hypoxia and ENT1 plays an important role in regulation of adenosine levels. As ENT1 inhibition was found neuroprotective (Cui *et al.,* 2013), its over activity was neurotoxic in our results and both

indicate from opposite ways that adenosine transported from extracellular to intracellular environment under acute hypoxic conditions and increases neurotoxicity.

Adenosine is a modulator on many brain functions that affects the outcome of brain injuries, stroke, ischemia and epileptic seizures (Di Angelantonio *et al.*, 2015; Pedata *et al.*, 2015). Among those conditions, stroke was preferred as an experimental model in our studies because of being an important health problem of humans and its strong relation to ARs.

In our first study, it was shown a detrimental effect of over expression of hENT1 in cortex which has abundant neuroprotective A_1Rs and less numbers of A_{2A}Rs are present. This finding was supported by our previous *in vitro* study that showed A_1R activity under glucose/oxygen deprivation was significantly lower in Tg hENT1 mice, relative to Wt, indicating that adenosine influx exceeds efflux at neuronal cell membranes (Chu *et al.*, 2014; Zhao *et al.*, 2011). We added *in vivo* evidence on this important finding by our study. Our results indicated that the main source of adenosine under stress might not be neurons but extraneuronal environment. Also it indicates that, during ischemia, neurons do not release adenosine via ENTs but actively salvage adenosine, which may be released by another mechanism, from another cell type, or formed extracellularly from released adenine nucleotides. There are some other literature that indicates the same results from different aspects. Cunha *et al.* (2005) showed ENT1 blockage increased extracellular adenosine concentrations in integrated brain preparations. In a recent study Cui *et al.*, (2013) also demonstrated that ENT1 inhibition by its antagonist slows down extracellular adenosine concentrations and it is neuroprotective. It was also demonstrated ENT1 null mice have decreased ischemia-reperfusion injury relative to Wt controls in a study (Rose *et al.*, 2010). In opposite way, by using hENT1 overexpression, we found increased cortical damage in our studies. All these together indicate, adenosine is more abundant in the

extracellular environment under stress and its uptake to intracellular area deprives the neurons from neuroprotective A_1Rs activity and causes more neurotoxicity. It should be remembered that during ischemic conditions, adenosine levels increase up to 100-fold (Frenguelli *et al.,* 2007; Parkinson *et al.* 2000) and faster removal of adenosine from extracellular environment could exaggerate the injury. However, lacking of microdialysis to measure extracellular adenosine concentrations is an important limitation in our studies.

Caffeine as a non-selective antagonist of ARs abolished the difference between Tg and Wt mice that indicated the specific role of ARs in stroke in our experiment. In the next step, we aimed to be more specific for ARs and used DPCPX, a selective A_1R antagonist. Histopathology was added to evaluate and to clarify whether MRI accurately determines the size of injured area. Thus, it was possible to observe the the success of establishing a stroke at microscopic level by details in necrosis, penumbra and surrounding tissue.

We also used a relatively simple and reproducible stroke model in our experiments although the age of mice in our experiment was not equal to elderly period in humans. There are many ways to establish a stroke models in rodents and some of them require invasive procedures (i.e., surgical or intraluminal monofilament occlusion of MCA (Kumar *et al.,* 2016; Braeuninger and Kleinschnitz, 2009, Shahjouei *et al.,* 2016)). Compared to invasive ones, we demonstrated a simple and relatively non-invasive technique in establishing stroke by direct injection of ET-1 to the cortex. Indeed, application of ET-1 directly to the cortex after a small burr hole opening provided sufficient vasoconstriction to produce a subsequent stroke. Since there are some controversial results in the literature about the efficacy of ET-1 injection to produce stroke in rodents, we believe that our findings might help in the efforts for selection of a better stroke model.

The role of A_1Rs in stroke is important from two points. First, A_1Rs are the most abundant ARs in neocortex which affects the motor functions. As it it is well known, adenosine produces its effect through a family of four G-protein coupled receptors, termed A_1, A_{2A}, A_{2B}, and A_3Rs. In brain, these receptors are located on neurons, astrocytes, microglia, and endothelial cells (Dunwiddie and Masino, 2001; Fredholm *et al.*, 2005). In contrast to abundant A_1Rs in the cortex, A_{2A}Rs are predominantly on neurons in the striatum, nucleus accumbens and olfactory tubercle (Cunha *et al.*, 1994). Second, A_1R activation has neuroprotective effects and can produce sedation, antinociception, anticonvulsant effects, bradycardia, vasoconstriction, bronchoconstriction, antidiuresis and decreased glomerular filtration, and an increase in insulin sensitivity (Ribeiro and Sebastião, 2010). Since our previous study showed a trend for increased neuronal damage by enhanced neuronal expression of ENT1 and caffeine abolished this effect, we used A_1R specific antagonist, DPCPX to see whether the effects of such a specific blockage in cortical area would alter A_1R related neuroprotection. DPCPX was delivered in the vehicle DMSO. Overall, there was a synergistic effect of ENT1 overexpression and A_1R blockage which is resulted in significantly larger infarct size by histopathologic assesment. In contrast to caffeine, genotype related changes were not disappeared after DPCPX injection. This can indicate that other ARs could also play a role in neuroprotection/neurotoxicity in cortex together with A_1Rs. However, this needs further studies for better understanding. We also noted potential neuroprotective effect of DMSO, which was reported before by other authors (Varma *et al.*, 2002; Paunescu *et al.*, 2009). DPCPX was given at 1 mg/kg IP 30 min prior to ET-1 injection. The dose was chosen based on a study that was done by Phillis (1995) who compared 0.1 mg/kg and 1 mg/kg of DPCPX and there was a significant increase in ischemia-induced damage in hippocampus by higher dose. However, it was also reported no effect of DPCPX at 3 mg/kg, but

an anxiogenic-like response developed at 6 mg/kg on in the elevated plus-maze in mice (Prediger *et al*. 2004).

Several studies indicate that modification of ARs, i.e., up/down regulation by using antagonists/agonists in short/long term periods results in different responses in brain functions or conditions associated with ARs. In addition, acute vs. chronic use of agonists/antagonists affect each AR in different way. As an example, chronic caffeine consumption cause up-regulation in A_1R but not in $A_{2A}R$ by (Riberio and Sebastiao, 2010). Chronic (but not acute) antagonist treatment attenuates brain injury, possibly by A_1R-mediated suppression of glutamate release and inhibition of excessive inflammatory cytokine production (Li *et al*., 2008). High dose antagonist influences ARs differently than lower doses (Conlay *et al.*, 1997). There are many studies that discuss variabilities in antagonist dose/duration and ontogeny of ARs. Thus, it could be possible to see opposite effects of the same agent in different dose or time use. Caffeine, although it blocks ARs, has been shown as a neuroprotective agent in extreme premature babies and in elderly peoples' neurodegenerative diseases (Cunha, 2005; Rivera-Oliver and Diaz-Rios, 2014; Chen *et al.*, 2010; Schmidt *et al.*, 2007). As we know, caffeine is the most widely consumed substance over the world. It is not clear and still is questionable if caffeine consumption, especially in higher dose, has any ontogenic effect on ARs and affects the metabolism of caffeine. It is also not clear, if any modification in ARs by caffeine consumption during the pregnancy might change as a consequence the ventilatory response of newborns. In the first two chapters, we have focused on stroke as known as an elderly age problem which related to ARs. In the last two chapters we have focused on apnea a major problem of early newborn people. The shared point of these two diverse conditions was caffeine, adenosine and its receptors.

Caffeine is rapidly and completely absorbed from the gastrointestinal tract with minimal first-pass effect and enters all body tissues (Grosso *et al.,* 2006, Aden, 2011). Caffeine is a non-selective antagonist of A_1R and $A_{2A}R$ (Aden, 2011). When preterm babies are treated with caffeine, higher plasma levels also inhibit A_3Rs and phosphodiesterases, $GABA_A$ receptors and results in Ca^{2+} release from affected site (Aden, 2011).

A_1R and $A_{2A}R$ expression could be detected earlier and they become functional later (Weaver, 1996; Aden, 2011; Rivkees and Wendler, 2011). Therefore, we performed WB and rt-PCR to see ARs at gene level and protein level. We found an increased A_1R expression in striatum in P0 pups after 1 g/l caffeine supplementation to their mothers during pregnancy period. Also there was an increased gene expression of both receptors at BS by caffeine exposure. This is very important, because BS is the main control center of breathing. So, we could suggest that caffeine exposure changes the ontogeny of ARs in the BS and in the medulla. However, this effect disappeared by P6. It should be remembered that P0 pups represent 24 weeks gestation in humans and P6 is equal to 32 weeks in newborn babies by brain development (Biran *et al.*, 2012). Apnea is an important problem of extreme preterm babies and it improves by advanced age. Our findings about increased gene expression of ARs in the BS indicates that exposure to caffeine could modify ontogeny of ARs. This opens the question if any extreme preterm baby behaves differently for respiratory drive if his/her mother consumes high doses of caffeine during the pregnancy. We tried to answer this question in the next chapter. However, it should also be confirmed by further respiratory studies in P0 pups and clinical observation in human newborns to see if they have less apnea than the others. Despite its blockade of ARs, caffeine is clinically neuroprotective in newborns perhaps by decreasing hypoxia numbers and duration (Schmidt *et al.,* 2007). However, we also found that A_1Rs in the striatum increases by

long term exposure to caffeine during pregnancy. Does this increase in A_1Rs also contribute the neuroprotection in extreme preterms born around 24 weeks? It could be very important to know the answer to this question by bench to bed study since there is no human or animal data about this effect in the literature.

There are studies reporting opposite results to our study by using a similar protocol. Exposure of pregnant rats to 1 g/l of caffeine during pregnancy resulted in an approximately 50% down-regulation in total A_1R but not in A_{2A}R (Leon *et al.,* 2002). Similar findings were found Lorenzo *et al.* (2010) and Kaplan *et al.* (1993). All these results indicate that the effect of caffeine exposure on AR abundance needs further studies since the results show big variations.

Another aim in our study was to elucidate if maternal caffeine intake during pregnancy affects neonatal caffeine metabolism. In current literature there is very limited information to answer this question. We observed a reliable level of plasma caffeine in P0 pups (5.32 ±2.03 µg/ml) by exposure to 92 ±31 mg/kg/day caffeine citrate during the gestation. But there was no difference in caffeine metabolism by P6. This finding also requires further studies to elucidate enzymatic changes, especially CYP 1A2, to understand whether this occurs at the molecular level.

Interestingly, we found a significant decrease in offspring numbers in rats receiving high dose of caffeine during pregnancy. Although caffeine`s effect on fertility is long lasting debate, the results are controversial (Aden, 2011; Okubo *et al.,* 2015). Adverse effects of caffeine on number of offspring were reported only in a few studies in animals. We believe detrimental effect of caffeine is multifactorial and is affected by caffeine protocol and stage or duration of pregnancy that was caffeine-exposed. Human data by clinical observations is more concerning. It was reported that caffeine consumption of >7 cups/day is associated with risk for decreased

fertility (Christianson *et al.,* 1989), spontaneous abortion, stillbirth and preterm delivery (Greenwood *et al.* 2014; Bech *et al.* 2005: Tolstrup *et al.* 2003).

Pups in the Cg had significant growth retardation compared to pups in the Wg in ours study. This finding has been supported by human and animal data (Jahanfar and Jaafar, 2015; Fujii *et al*. 1972; Elmazar *et al.*1982; Brent *et al.*2011). Based on these results, caffeine intake during the pregnancy has been advised to be limited to 200 mg/day immediately before and during pregnancy (Greenwood *et al.,* 2014; http://cot.food.gov.uk, 2008).

Maternal caffeine consumption may have long-term consequences and effects on respiratory control systems in their offspring (Cayetanot *et al.,* 2009). We attempted to exaggerate a hypoxic biphasic ventilatory response, a stimulation followed by a depression as described by other authors (Pamenter *et al.,* 2015, Koos *et al.,* 2004, Zhao *et al.,* 2011, Picard *et al.,* 2008). In our study, hypoxia caused respiratory depression in RR and MV regardless of whether the respiratory stimulant caffeine was adminstered. TV has appeared as a protective mechanism against this depression and showed a compensatory increase over time. We could not count apneas in our study due to excessive artefacts on respiratory waves due to movements of rat pups in plethysmograph chambers. Repeated caffeine exposure during intrauterine and extrauterine life caused more depressive effect on RR compared to other groups, but it was compensated by protected TV probably effect of caffeine on respiratory muscles.

From our results, caffeine has limited ontogenical effect of AR development and it can affect respiratory drive under hypoxia if any newborn is exposed to caffeine both intrauterine and extrauterine periods. But this observation is very important when considering sudden infant death was higher in mothers who consumed excessive amount of caffeine during their pregnancy and lactation. Further studies with sophisticated technology to include apnea in these parameters

will provide for better connection of our findings to clinical area. Also, it should be remembered that ARs are not the only target for caffeine. Therefore, further efforts should include other caffeine related systems such as dopaminergic, serotoninergic and GABAergic receptors in further studies.

Chapter VII:

References

Abdi F, Pollard I, Wilkinson J (1993). Placental transfer and foetal disposition of caffeine and its immediate metabolites in the 20-day pregnant rat: function of dose. *Xenobiotica* **23:**449-456.

Adén U, Herlenius E, Tang LQ, Fredholm BB (2000). Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. *Pediatr Res* **48:**177-183.

Adén U (2011). Methylxanthines during pregnancy and early postnatal life. *Handb Exp Pharmacol* **200:**373-389.

Albensi BC, Schweizer MP, Rarick TM, Filloux F (1999). Unilateral hypoxic-ischemic injury in the neonatal rat brain evaluated by *in vivo* MRI. Correlation with histopathology and neuroprotection by MK-801. *Invest Radiol* **34:**249-261.

Albensi BC, Schweizer MP, Rarick TM, Filloux F (1998). Magnetic resonance imaging of hypoxic-ischemic brain injury in the neonatal rat. *Invest Radiol* **33**:377-385.

Anderson CM, Xiong W, Geiger JD, Young JD, Cass CE, Baldwin SA, Parkinson FE (1999). Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. *J Neurochem* **73**:867-873.

Aranda JV, Beharry K, Valencia GB, Natarajan G, Davis J (2010). Caffeine impact on neonatal morbidities. *J Matern Fetal Neonatal Med* **23** Suppl **3:**20-23.

Aranda JV, Collinge JM, Zinman R, Watters G (1979). Maturation of caffeine elimination in infancy. *Arch Dis Child* **54:**946-949.

Arnaud MJ (2011). Pharmacokinetics and metabolism of natural methylxanthines in animal and

man. *Handb Exp Pharmacol* **200:**33-91.

Bacigaluppi M, Comi G, Hermann DM (2010). Animal models of ischemic stroke. Part two: modeling cerebral ischemia. *Open Neurol J* **4:**34-38.

Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD (2004). The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* **447:**735-743.

Bech BH, Nohr EA, Vaeth M, Henriksen TB, Olsen J. (2005). Coffee and fetal death: a cohort study with prospective data. *American Jornal of Epidemiology* **162:**983-990.

Bhatt-Mehta V, Schumacher RE (2003). Treatment of apnea of prematurity. *Paediatric Drugs* **5:**195-210.

Biran V, Verney C, Ferriero DM (2012). Perinatal cerebellar injury in human and animal models. *Neurol Res Int* **12:**858-929

Bjorklund O, Shang M, Tonazzini I, Daré E, Fredholm BB (2008). Adenosine A1 and A3 receptors protect astrocytes from hypoxic damage. *Eur J Pharmacol* **596:**6-13.

Bodenmann S, Hohoff C, Freitag C, Deckert J, Rétey JV, Bachmann V, Landolt HP (2012). Polymorphisms of ADORA2A modulate psychomotor vigilance and the effects of caffeine on neurobehavioural performance and sleep EEG after sleep deprivation. *Br J Pharmacol* **165:**1904-1913.

Bodineau L, Saadani-Makki F, Jullien H, Frugière A (2006). Caffeine in the milk prevents respiratory disorders caused by in utero caffeine exposure in rats. *Respir Physiol Neurobiol* **150:**94-98.

Bodineau L, Cayetanot F, Sådani-Makki F, Bach V, Gros F, Lebleu A, Collin T, Frugière A (2003). Consequences of in utero caffeine exposure on respiratory output in normoxic and hypoxic conditions and related changes of Fos expression: a study on brainstem-

spinal cord preparations isolated from newborn rats. *Pediatr Res* **53:**266-273.

Boison D, Chen JF, Fredholm BB (2010). Adenosine signaling and function in glial cells. Cell Death Differ. **17:**1071-1082.

Bona E, Adén U, Fredholm BB, Hagberg H (1995). The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. *Pediatric Research* **38:**312-318.

Borea PA, Gessi S, Merighi S, Varani K (2016). Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? *Trends Pharmacol Sci* **37:**419-434.

Borea PA, Varani K, Vincenzi F, Baraldi PG, Tabrizi MA, Merighi S, Gessi S (2014). The A3 adenosine receptor: history and perspectives. *Pharmacol Rev* **67:7**4-102.

Borea PA, Gessi S, Bar-Yehuda S, Fishman P (2009). A3 adenosine receptor: pharmacology and role in disease. *Handb Exp Pharmacol* **193:**297-327.

Boulenger JP, Patel J, Post RM, Parma AM, Marangos PJ (1983). Chronic caffeine consumption increases the number of brain adenosine receptors. *Life Sciences* **32:**1135–1142.

Braeuninger S, Kleinschnitz C (2009). Rodent models of focal cerebral ischemia: procedural pitfalls and translational problems. *Exp Transl Stroke Med* **25:**1-8.

Braun N, Lenz C, Gillardon F, Zimmermann M, Zimmermann H (1997). Focal cerebral ischemia enhances glial expression of ecto-5'-nucleotidase. *Brain Res* **766:**213-226.

Brent RL, Christian MS Diener RM (2011). Evaluation of the reproductive and developmental risks of caffeine. Birth defects research. Part B, *Developmental and reproductive toxicology* **92:**152-187.

Browne ML (2006). Maternal exposure to caffeine and risk of congenital anomalies: a systematic review. *Epidemiology* **17:**324-331.

Burnstock G (1972). Purinergic nerves. *Pharmacol Rev* **24:**509-581.

Burnstock G (1976). Purinergic receptors. *J Theor Biol* **21:**491-503.

Buters JT, Tang BK, Pineau T, Gelboin HV, Kimura S, Gonzalez FJ (1996). Role of CYP1A2 in caffeine pharmacokinetics and metabolism: studies using mice deficient in CYP1A2. *Pharmacogenetics* **6:**291-296.

Cassada DC, Tribble CG, Young JS, Gangemi JJ, Gohari AR, Butler PD, Rieger JM, Kron IL, Linden J, Kern JA (2002). Adenosine A2A analogue improves neurologic outcome after spinal cord trauma in the rabbit. *J Trauma* **53:**225-229.

Cayetanot F, Larnicol N, Peyronnet J (2009). Antenatal environmental stress and maturation of the breathing control, experimental data. *Respir Physiol Neurobiol* **168:**92-100.

CARE Study Group (2008). Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. *BMJ* **337:**a2332.

Chang YS, Mu D, Wendland M, Sheldon RA, Vexler ZS, McQuillen PS, Ferriero DM (2005). Erythropoietin improves functional and histological outcome in neonatal stroke. *Pediatr Res* **58:**106-111.

Chen LW, Wu Y, Neelakantan N, Chong MF, Pan A, van Dam RM (2014). Maternal caffeine intake during pregnancy is associated with risk of low birth weight: a systematic review and dose-response meta-analysis. *Biomed Central Medicine* **19:**174.

Chen JF, Yu L, Shen HY, He JC, Wang X, Zheng R (2010). What knock-out animals tell us about the effects of caffeine. *J Alzheimers Dis* **20:**S17-24.

Chen X, Gawryluk JW, Wagener JF, Ghribi O, Geiger JD (2008). Caffeine blocks disruption of blood brain barrier in a rabbit model of Alzheimer's disease. *J Neuroinflammation* **5:**12.

Chen J, Rinaldo L, Lim SJ, Young H, Messing RO, Choi DS (2007). The type 1 equilibrative

nucleoside transporter regulates anxiety-like behavior in mice. *Genes Brain Behav.* **6:**776-783.

Christianson RE, Oechsli FW, van den Berg BJ (1989). Caffeinated beverages and decreased fertility. *Lancet* **8634:**378.

Chu S, Xiong W, Zhang D, Soylu H, Sun C, Albensi BC, Parkinson FE (2013). Regulation of adenosine levels during cerebral ischemia. *Acta Pharmacol Sin* **34**:60-66.

Chu S, Xiong W, Parkinson FE (2014). Effect of ecto-5'-nucleotidase (eN) in astrocytes on adenosine and inosine formation. *Purinergic Signalling* **10**:603-609.

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) (2008). COT statement on the reproductive effects of caffeine. http://cot.food.gov.uk/cotstatements/cotstatementsyrs/cotstatements2008/cot200804; Accessed 29 March 2015.

Comer AM, Perry CM, Figgitt DP (2001). Caffeine citrate: a review of its use in apnoea of prematurity. *Paediatr Drugs* **3:**61-79.

Conde SV, Monteiro EC, Obeso A, Gonzalez C (2009).Adenosine in peripheral chemoreception :new insights into a historically overlooked molecule: invited article. *Adv Exp Med Biol* **648:**145-159.

Conlay LA, Conant JA, deBros F, Wurtman R (1997). Caffeine alters plasma adenosine levels. *Nature* **389:**136.

Costenla AR, Cunha RA, de Mendonça A (2010). Caffeine, adenosine receptors, and synaptic plasticity. *J Alzheimers Dis* **20:**S25-34.

Corbet AJ, Flax P, Alston C, Rudolph AJ (1978). Effect of aminophylline and dexamethasone on secretion of pulmonary surfactant in fetal rabbits. *Pediatr Res* **12:**797-799.

Cui M, Bai X, Li T, Chen F, Dong Q, Zhao Y, Liu X (2013). Decreased extracellular adenosine levels lead to loss of hypoxia-induced neuroprotection after repeated episodes of exposure to hypoxia. *PLoS One* **8(2):**e57065.

Cunha RA (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int* **38:**107-125.

Cunha RA (2005). Neuroprotection by adenosine in the brain: From A(1) receptor activation to A(2A) receptor blockade. *Purinergic Signal* **1:**111-134.

Cunha RA, Johansson B, van der Ploeg I, Sebastiao AM, Ribeiro JA, Fredholm BB (1994). Evidence for functionally important adenosine A2a receptors in the rat hippocampus *Brain Res* **649:**208–216.

Dagal A, Lam AM (2011). Cerebral blood flow and the injured brain: how should we monitor and manipulate it? *Curr Opin Anaesthesiol* **24:**131-137.

Dale N, Frenguelli BG (2009). Release of adenosine and ATP during ischemia and epilepsy. *Curr Neuropharmacol* **7:**160-179.

Daly JW (2007). Caffeine analogs: biomedical impact. *Cell Mol Life Sci* **64:**2153-2169.

Deb P, Sharma S, Hassan KM (2010). Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology* **17:**197-218.

Desfrere L, Olivier P, Schwendimann L, Verney C, Gressens P (2007). Transient inhibition of astrocytogenesis in developing mouse brain following postnatal caffeine exposure. *Pediatr Res* **62:**604-609.

Detre JA, Wang J, Wang Z, Rao H (2009). Arterial spin-labeled perfusion MRI in basic and

clinical neuroscience. *Curr Opin Neurol* **22:**348-355.

Di Angelantonio S, Bertollini C, Piccinin S, Rosito M, Trettel F, Pagani F, Limatola C, Ragozzino D (2015). Basal adenosine modulates the functional properties of AMPA receptors in mouse hippocampal neurons through the activation of A1R A2AR and A3R. *Front Cell Neurosci* **9:**409.

Dijkhuizen RM (2011). Advances in MRI-based detection of cerebrovascular changes after experimental traumatic brain injury. *Transl Stroke Res* **2:**524-532.

Drury AN, Szent-Gyorgyi A (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol* **68:**213-237.

Dunwiddie TV, Diao L, Kim HO, Jiang JL, Jacobson KA (1997). Activation of hippocampal adenosine A3 receptors produces a desensitization of A1 receptor-mediated responses in rat hippocampus. *J Neurosci* **17:**607-614.

Dunwiddie TV, Masino SA (2001). The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* **24:**31-55.

Durukan A, Tatlisumak T (2007). Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacol Biochem Behav* **87:**179-197.

Elmazar MM, McElhatton PR, Sullivan FM (1982). Studies on the teratogenic effects of different oral preparations of caffeine in mice. *Toxicology* **23:**57-71.

Evans MC, Swan JH, Meldrum BS (1987). An adenosine analogue, 2-chloroadenosine, protects against long term development of ischaemic cell loss in the rat hippocampus. *Neurosci. Lett.* **83:**287-292.

Fastbom J, Pazos A, Palacios JM (1987). The distribution of adenosine A1 receptors and 5'-

nucleotidase in the brain of some commonly used experimental animals. *Neuroscience.* **22:** 813-826.

Fredholm BB (1982). Adenosine actions and adenosine receptors after 1 week treatment with caffeine. *Acta Physiologica Scandinavica* **115:**283-286.

Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* **51:**83-133.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005). Adenosine and brain function. *Int Rev Neurobiol* **63:**191-270.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53:**527–552

Frenguelli BG, Wigmore G, Llaudet E, Dale N (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J Neurochem* **101:**1400-1413.

Fricker J (2000). Unravelling adenosine's effects on stroke. *Mol Med Today* **6:**48.

Fujii T, Nishimura H (1972). Adverse effects of prolonged administration of caffeine on rat fetus. *Toxicol Appl Pharmacol* **22:**449-457.

Fuxe K, Bjelke B, Andbjer B, Grahn H, Rimondini R, Agnati LF (1997). Endothelin-1 induced lesions of the frontoparietal cortex of the rat: A possible model of focal cortical ischemia. *Neuroreport.* **8:**2623–2629.

Fuxe K, Cintra A, Andbjer B, Anggård E, Goldstein M, Agnati LF (1989). Centrally administered endothelin-1 produces lesions in the brain of the male rat. *Acta Physiol*

Scand **137:** 155-156.

Gaytan SP, Pasaro R (2012). Neonatal caffeine treatment up-regulates adenosine receptors in brainstem and hypothalamic cardio-respiratory related nuclei of rat pups. *Experimental Neurology* **237:**247-259.

Gaytan SP, Saadani-Makki F, Bodineau L, Frugière A, Larnicol N, Pásaro R (2006). Effect of postnatal exposure to caffeine on the pattern of adenosine A1 receptor distribution in respiration-related nuclei of the rat brainstem. *Auton Neurosci* **126-127:**339-346.

George L, Granath F, Johansson AL, Olander B, Cnattingius S (2006). Risks of repeated miscarriage. *Paediatric and Perinatal Epidemiology* **20:**119-126.

Gessi S, Merighi S, Varani K, Borea PA (2011). Adenosine receptors in health and disease. *Adv Pharmacol* **61:**41-75.

Giannelli M, Doyle P, Roman E, Pelerin M, Hermon C (2003). The effect of caffeine consumption and nausea on the risk of miscarriage. *Paediatr Perinat Epidemiol* **17:**316-323.

Gilmour G, Iversen SD, O'Neill MF, Bannerman DM (2004). The effects of intracortical endothelin-1 injections on skilled forelimb use: implications for modeling recovery of function after stroke. *Behav Brain Res* **150:**171-183.

Gray JH, Owen RP, Giacomini KM (2004). The concentrative nucleoside transporter family, SLC28. *Pflugers Arch* **447:**728-734.

Green RM, Stiles GL (1986). Chronic caffeine ingestion sensitizes the A1 adenosine receptor-adenylate cyclase system in rat cerebral cortex. *J Clin Invest* **77:**222-227.

Greenwood DC, Thatcher NJ, Ye J, Garrard L, Keogh G, King LG, Cade JE (2014). Caffeine intake during pregnancy and adverse birth outcomes: a systematic review and dose-

response meta-analysis. *Eur J Epidemiol* **29:**725-734.

Grosso LM, Triche EW, Belanger K, Benowitz NL, Holford TR, Bracken MB (2006). Caffeine metabolites in umbilical cord blood, cytochrome P-450 1A2 activity, and intrauterine growth restriction. *American Journal of Epidemiology* **163:**1035-1041.

Gui L, Duan W, Tian H, Li C, Zhu J, Chen JF, Zheng J (2009). Adenosine A2A receptor deficiency reduces striatal glutamate outflow and attenuates brain injury induced by transient focal cerebral ischemia in mice. *Brain Res* **1297:**185-193.

Guillet R, Kellogg C (1991). Neonatal exposure to therapeutic caffeine alters the ontogeny of adenosine A1 receptors in brain of rats. *Neuropharmacology* **30:**489-496.

Hahn KA, Wise LA, Rothman KJ, Mikkelsen EM, Brogly SB, Sørensen HT, Riis AH, Hatch EE (2015) Caffeine and caffeinated beverage consumption and risk of spontaneous abortion. *Hum Reprod* **30:**1246-1255.

Henderson-Smart DJ, De Paoli AG (2010). Methylxanthine treatment for apnoea in preterm infants. *Cochrane Database Syst Rev* :CD000140.

Heitzmann D, Buehler P, Schweda F, Georgieff M, Warth R, Thomas J (2016). The *in vivo* respiratory phenotype of the adenosine A1 receptor knockout mouse. *Respir Physiol Neurobiol* **222:**16-28.

Hentges CR, Guedes RR, Silveira RC, Procianoy RS (2010). Serum levels of caffeine in umbilical cord and apnea of prematurity. *J Pediatr (Rio J)* **86:**137-142.

Herlenius E, Adén U, Tang LQ, Lagercrantz H (2002). Perinatal respiratory control and its modulation by adenosine and caffeine in the rat. *Pediatr Res* **51**:4-12.

Hoecker C, Nelle M, Poeschl J, Beedgen B, Linderkamp O (2002). Caffeine impairs cerebral and intestinal blood flow velocity in preterm infants. *Pediatrics* **109:**784-787.

Hoehn M, Nicolay K, Franke C, van der Sanden B (2001). Application of magnetic resonance to animal models of cerebral ischemia. *J Magn Reson Imaging* **14:**491-509.

Holtzman SG, Mante S, Minneman KP (1991). Role of adenosine receptors in caffeine tolerance. *Journal of Pharmacology and Experimental Therapeutics* **256:**62–68

Horie N, Maag AL, Hamilton SA, Shichinohe H, Bliss TM, Steinberg GK (2008). Mouse model of focal cerebral ischemia using endothelin-1. *J Neurosci Methods* **173:**286-290.

Hoyt AT, Browne M, Richardson S, Romitti P, Druschel C (2014). National Birth Defects Prevention Study. Maternal caffeine consumption and small for gestational age births: results from a population-based case-control study. *Maternal and Child Health Journal* **18:**1540-1551.

Huang ZL, Zhang Z, Qu WM (2014). Roles of adenosine and its receptors in sleep-wake regulation. *Int Rev Neurobiol* **119:**349-371.

Jacobson KA, von Lubitz DK, Daly JW, Fredholm BB (1996). Adenosine receptor ligands: differences with acute versus chronic treatment. *Trends Pharmacol Sci* **17:**108-113.

Jacobson KA (2009). Introduction to adenosine receptors as therapeutic targets. *Handb Exp Pharmacol* **193:**1-24.

Jacobson KA, Gao ZG (2009). Adenosine. In: Squire LR (ed.) *Encyclopedia of Neuroscience*, Oxford: Academic Press **1:**83-95.

Jahanfar S, Jaafar SH (2015). Effects of restricted caffeine intake by mother on fetal, neonatal and pregnancy outcomes. *Cochrane Database Syst Rev* **9;(6):**CD006965.

James JE (2015). Review: higher caffeine intake during pregnancy increases risk of low birth weight. *Evid Based Nurs* **18:**111.

James JE, Paull I (1985). Caffeine and human reproduction. *Reviews on Environmental Health* **5:**

151-167.

Janjua N, Mayer SA (2003). Cerebral vasospasm after subarachnoid hemorrhage. *Curr Opin Crit Care* **9:**113-119.

Jennings LL, Hao C, Cabrita MA, Vickers MF, Baldwin SA, Young JD, Cass CE (2001). Distinct regional distribution of human equilibrative nucleoside transporter proteins 1 and 2 (hENT1 and hENT2) in the central nervous system. *Neuropharmacology* **40:**722-731.

Joesoef MR, Beral V, Rolfs RT, Aral SO, Cramer DW (1990). Are caffeinated beverages risk factors for delayed conception? *Lancet* **335:**136-137.

Johansson B, Ahlberg S, van der Ploeg I, Brene S, Lindefors N, Persson H, Fredholm BB (1993). Effect of long term caffeine treatment on A1 and A2 adenosine receptor binding and on mRNA levels in rat brain. *Naunyn Schmiedeberg's Archives of Pharmacology* **347:**407–414.

Julien CA (2011). Alteration of carotid body chemoreflexes after neonatal intermittent hypoxia and caffeine treatment in rat pups. *Respir Physiol Neurobiol* **177:**301-312.

Julien CA, Joseph V, Bairam A (2010). Caffeine reduces apnea frequency and enhances ventilatory long-term facilitation in rat pups raised in chronic intermittent hypoxia. *Pediatr Res* **68:**105-111.

Julien C, Bairam A, Joseph V (2008). Chronic intermittent hypoxia reduces ventilatory long-term facilitation and enhances apnea frequency in newborn rats. *Am J Physiol Regul Integr Comp Physiol* **294:**R1356-1366.

Karcz-Kubicha M, Antoniou K, Terasmaa A, Quarta D, Solinas M, Justinova Z, Pezzola A, Reggio R, Müller CE, Fuxe K, Goldberg SR, Popoli P, Ferré S (2003). Involvement of

adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. *Neuropsychopharmacology* **28:**1281-1291.

Kallur T, Hoehn M (2011). Experimental stroke research: the contributions of *in vivo* MRI. *Methods Mol Biol* **771:**255-275.

Kaplan GB, Greenblatt DJ, Kent MA, Cotreau-Bibbo MM (1993). Caffeine treatment and withdrawal in mice: relationships between dosage, concentrations, locomotor activity and A1 adenosine receptor binding. *J Pharmacol Exp Ther* **266:**1563-1572.

Karypidis AH, Söderström T, Nordmark A, Granath F, Cnattingius S, Rane A (2006). Association of cytochrome P450 1B1 polymorphism with first-trimester miscarriage. *Fertil Steril* **86:**1498-1503.

Kitagawa H, Mori A, Shimada J, Mitsumoto Y, Kikuchi T (2002). Intracerebral adenosine infusion improves neurological outcome after transient focal ischemia in rats. *Neurol Res* **24:**317-323.

Koos BJ, Kawasaki Y, Kim YH, Bohorquez F (2004). Adenosine A2A-receptor blockade abolishes the roll-off respiratory response to hypoxia in awake lambs. *Am J Physiol Regul Integr Comp Physiol* **288:** R1185-1194.

Koos BJ, Maeda T, Jan C (2001). Adenosine A1 and A2A receptors modulate sleep state and breathing in fetal sheep. *J Appl Physiol* **91:** 343–350.

Kost S, Sun C, Xiong W, Graham K, Cass CE, Young JD, Albensi BC, Parkinson FE (2011). Behavioral effects of elevated expression of human equilibrative nucleoside transporter 1 in mice. *Behav Brain Res* **224:**44-49.

Kotsopoulos J, Eliassen AH, Missmer SA, Hankinson SE, Tworoger SS (2009). Relationship between caffeine intake and plasma sex hormone concentrations in premenopausal and

postmenopausal women. *Cancer* **115:** 2765-2774.

Kren R, Ogushi S, Miyano T (2004). Effect of caffeine on meiotic maturation of porcine oocytes. *Zygote* **12:**31-38.

Kumar A, Aakriti, Gupta V (2016). A review on animal models of stroke: An update. *Brain Res Bull* **122:**35-44.

Kusano Y, Echeverry G, Miekisiak G, Kulik TB, Aronhime SN, Chen JF, Winn HR (2010). Role of adenosine A2 receptors in regulation of cerebral blood flow during induced hypotension. *J Cereb Blood Flow Metab* **30:**808-815.

Latini S, Corsi C, Pedata F, Pepeu G (1995). The source of brain adenosine outflow during ischemia and electrical stimulation. *Neurochem Int* **27:**239-244.

León D, Albasanz JL, Ruíz MA, Fernández M, Martín M (2002). Adenosine A1 receptor down-regulation in mothers and fetal brain after caffeine and theophylline treatments to pregnant rats. *J Neurochem* **82:**625-634.

Li W, Dai S, An J, Li P, Chen X, Xiong R, Liu P, Wang H, Zhao Y, Zhu M, Liu X, Zhu P, Chen JF, Zhou Y (2008). Chronic but not acute treatment with caffeine attenuates traumatic brain injury in the mouse cortical impact model. *Neuroscience* **151:**1198-1207.

Lin CL, Su YF, Dumont AS, Shih HC, Lieu AS, Howng SL, Lee KS, Kwan AL (2006). The effect of an adenosine A1 receptor agonist in the treatment of experimental subarachnoid hemorrhage-induced cerebrovasospasm. *Acta Neurochir* **148:**873-879.

Lopes LV, Halldner L, Rebola N, Johansson B, Ledent C, Chen JF, Fredholm BB, Cunha RA (2004). Binding of the prototypical adenosine A(2A) receptor agonist CGS 21680 to the cerebral cortex of adenosine A(1) and A(2A) receptor knockout mice. *Br J Pharmacol* **141:**1006-1014.

Lorenzo AM, León D, Castillo CA, Ruiz MA, Albasanz JL, Martín M (2010). Maternal caffeine intake during gestation and lactation down-regulates adenosine A1receptor in rat brain from mothers and neonates. *Journal of Neuroscience Research* **88:**1252-1261.

Lu H, Chen C, Klaassen C (2004). Tissue distribution of concentrative and equilibrative nucleoside transporters in male and female rats and mice. *Drug Metab Dispos* **32:**1455-1461.

Luo H, Deng Z, Liu L, Shen L, Kou H, He Z, Ping J, Xu D, Ma L, Chen L, Wang H (2014). Prenatal caffeine ingestion induces transgenerational neuroendocrine metabolic programming alteration in second generation rats. *Toxicol Appl Pharmacol* **274:** 383-392.

Luo J, Grammas P (2010). Endothelin-1 is elevated in Alzheimer's disease brain microvessels and is neuroprotective. *J Alzheimers Dis* **21:**887-896.

Markus H (2003). Acute stroke. *Int Psychogeriatr* **S1:**161-166.

Martin RJ, Wilson CG, Abu-Shaweesh JM, Haxhiu MA (2004). Role of inhibitory neurotransmitter interactions in the pathogenesis of neonatal apnea: implications for management. *Semin Perinatol* **28:**273-278.

Martin ED, Fernandez M, Perea G, Pascual O, Haydon PG, Araque A, Cena V (2007a). Adenosine released by astrocytes contributes to hypoxia-induced modulation of synaptic transmission. *Glia* **55:**36-45.

Martín I, López-Vílchez MA, Mur A, García-Algar O, Rossi S, Marchei E, Pichini S (2007b). Neonatal withdrawal syndrome after chronic maternal drinking of mate. *Ther Drug Monit* **29:**127-129

Matsuoka I, Ohkubo S (2004). ATP- and adenosine-mediated signaling in the central nervous system: adenosine receptor activation by ATP through rapid and localized generation of

adenosine by ecto-nucleotidases. *J Pharmacol Sci* **94:**95-99.

McGowan JD, Altman RE, Kanto WP Jr (1988). Neonatal withdrawal symptoms after chronic maternal ingestion of caffeine. *South Med J* **81:**1092-1094.

Melani A, Pugliese AM, Pedata F (2014). Adenosine receptors in cerebral ischemia. *Int Rev Neurobiol* **119:**309-348.

Melani A, Cipriani S, Vannucchi MG, Nosi D, Donati C, Bruni P, Giovannini MG, Pedata F (2009). Selective adenosine A2a receptor antagonism reduces JNK activation in oligodendrocytes after cerebral ischaemia. *Brain* **132:**1480-1495.

Meng S, Qiao M, Scobie K, Tomanek B, Tuor UI (2006). Evolution of magnetic resonance imaging changes associated with cerebral hypoxia-ischemia and a relatively selective white matter injury in neonatal rats. *Pediatr Res* **59:**554-559.

Minelli A, Bellezza I (2011). Methylxanthines and reproduction. *Handb Exp Pharmacol* **200:**349-72.

Mohamed RA, Agha AM, Abdel-Rahman AA, Nassar NN (2016). Role of adenosine A2A receptor in cerebral ischemia reperfusion injury: Signaling to phosphorylated extracellular signal-regulated protein kinase (pERK1/2). *Neuroscience* **314:**145-59.

Mohan A, Roberto AJ, Mohan A, Liogier-Weyback L, Guha R, Ravishankar N, Rebello C, Kumar A, Mohan R (2015). Caffeine as Treatment for Alzheimer's Disease: A Review *J Caffeine Res* **5:** 61-64.

Molina-Arcas M, Trigueros-Motos L, Casado FJ, Pastor-Anglada M (2008). Physiological and pharmacological roles of nucleoside transporter proteins. *Nucleosides Nucleotides Nucleic Acids* **27:**769-778.

Montandon G, Bairam A, Kinkead R (2006). Long-term consequences of neonatal caffeine on

ventilation, occurrence of apneas, and hypercapnic chemoreflex in male and female rats. *Pediatr Res* **59:**519-524.

Montandon G, Kinkead R, Bairam A (2007). Disruption of adenosinergic modulation of ventilation at rest and during hypercapnia by neonatal caffeine in young rats: role of adenosine A(1) and A(2A) receptors. *Am J Physiol Regul Integr Comp Physiol* **292:**R1621-631.

Montandon G, Kinkead R, Bairam A (2008). Adenosinergic modulation of respiratory activity: developmental plasticity induced by perinatal caffeine administration. *Respir Physiol Neurobiol* **164:**87-95.

Mosca EV, Ciechanski P, Roy A, Scheibli EC, Ballanyi K, Wilson RJ (2014). Methylxanthine reversal of opioid-induced respiratory depression in the neonatal rat: mechanism and location of action. *Respiratory Physiology and Neurobiology* **200:**80-89.

Müller CE, Jacobson KA (2011). Recent developments in adenosine receptor ligands and their potential as novel drugs. *Biochim Biophys Acta* **1808:**1290-308.

Natarajan G, Botica ML, Thomas R, Aranda JV (2007). Therapeutic drug monitoring for caffeine in preterm neonates: an unnecessary exercise? *Pediatrics* **119:**936-940.

Nehlig A, Debry G (1994). Potential teratogenic and neurodevelopmental consequences of coffee and caffeine exposure: a review on human and animal data. *Neurotoxicol Teratol* **16:**531-543.

Nehlig A (2016). Effects of coffee/caffeine on brain health and disease: What should I tell my patients? *Pract Neurol* **16:**89-95.

Neumann RP, von Ungern-Sternberg BS (2014). The neonatal lung--physiology and ventilation. *Paediatr Anaesth* **24:**10-21.

Ngai AC, Coyne EF, Meno JR, West GA, Winn HR (2001). Receptor subtypes mediating adenosine-induced dilation of cerebral arterioles. *Am J Physiol Heart Circ Physiol* **280:**2329-35.

Nguyen MD, Ross AE, Ryals M, Lee ST, Venton BJ (2015). Clearance of rapid adenosine release is regulated by nucleoside transporters and metabolism. *Pharmacol ResPerspect* **3:**e00189.

O'Bryant Z, Vann KT, Xiong ZG (2014). Translational strategies for neuroprotection in ischemic stroke--focusing on acid-sensing ion channel 1a. *Transl Stroke Res* **5:**59-68.

Okada M, Nutt DJ, Murakami T, Zhu G, Kamata A, Kawata Y, Kaneko S (2001). Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. *J Neurosci* **21:**628-640.

Okubo H, Miyake Y, Tanaka K, Sasaki S, Hirota Y (2015). Maternal total caffeine intake, mainly from Japanese and Chinese tea, during pregnancy was associated with risk of preterm birth: the Osaka Maternal and Child Health Study. *Nutrition Research* **35:** 309-316.

Pamenter ME, Dzal YA, Milsom WK (2015). Adenosine receptors mediate the hypoxic ventilatory response but not the hypoxic metabolic response in the naked mole rat during acute hypoxia. *Proc R Soc B* **282:** 20141722.

Pan HZ, Chen HH (2007). Hyperalgesia, low-anxiety, and impairment of avoidance learning in neonatal caffeine-treated rats. *Psychopharmacology.* **191**:119-25.

Parkinson FE, Damaraju VL, Graham K, Yao SYM, Baldwin SA, Cass CE, Young JD (2011). Molecular Biology of nucleoside transporters and their distributions and functions in the brain. *Curr Top Med Chem* **11**:948-972.

Parkinson FE, Xiong W (2004). Stimulus- and cell-type-specific release of purines in cultured rat forebrain astrocytes and neurons. *J Neurochem* **88:**1305-1312.

Parkinson FE, Xiong W, Zamzow CR (2005). Astrocytes and neurons: Different roles in regulating adenosine levels. *Neurol Res* **27**:153-160.

Parkinson FE, Xiong W, Zamzow CR, Chestley T, Mizuno T, Duckworth ML (2009). Transgenic expression of human equilibrative nucleoside transporter 1 in mouse neurons. *J Neurochem* **109:**562-572.

Parkinson FE, Zhang YW, Shepel PN, Greenway SC, Peeling J, Geiger JD (2000). Effects of nitrobenzylthioinosine on neuronal injury, adenosine levels, and adenosine receptor activity in rat forebrain ischemia. *J Neurochem* **75:**795-802.

Paunescu H, Coman OA, Panaitescu E, Anca A, Ghita I, Fulga I (2009). Experimental study on the analgesic effect of dimethylsulfoxide in mice using the writhing test.*Therap Pharmacol Clin Toxicol* **13:**270-272.

Paxinos G, Franklin KBJ (2001). The mouse brain in stereotaxic coordinates. 2nd edition. San Diego: Academic Press.

Pedata F, Dettori I, Coppi E, Melani A, Fusco I, Corradetti R, Pugliese AM (2016). Purinergic signalling in brain ischemia. *Neuropharmacology* **104:**105-130.

Peeling J, Corbett D, Del Bigio MR, Hudzik TJ, Campbell TM, Palmer GC (2001). Rat middle cerebral artery occlusion: correlations between histopathology, T2-weighted magnetic resonance imaging, and behavioral indices. *J Stroke Cerebrovasc Dis* **10:**166-177.

Pelligrino DA, Xu HL, Vetri F (2010). Caffeine and the control of cerebral hemodynamics. *J Alzheimers Dis* **20 Suppl 1:**S51-62.

Phillis JW (1995). The effects of selective A1 and A2a adenosine receptor antagonists on

cerebral ischemic injury in the gerbil. *Brain Res* **705:**79-84.

Picard N, Guénin S, Larnicol N, Perrin Y (2008). Maternal caffeine ingestion during gestation and lactation influences respiratory adaptation to acute alveolar hypoxia in newborn rats and adenosine A2A and GABA A receptor mRNA transcription. *Neuroscience* **156:**630-639.

Pignataro G, Simon RP, Boison D (2007). Transgenic overexpression of adenosine kinase aggravates cell death in ischemia. *J Cereb Blood Flow Metab* **27:**1-5.

Pollard I, Jabbour H, Mehrabani PA (1987). Effects of caffeine administered during pregnancy on fetal development and subsequent function in the adult rat: prolonged effects on a second generation. *J Toxicol Environ Health* **22:**1-15.

Porkka-Heiskanen T, Kalinchuk AV (2011). Adenosine, energy metabolism and sleep homeostasis. *Sleep Med Rev* **15:**123–135.

Powell FL, Milsom WK, Mitchell GS (1998). Time domains of the hypoxic ventilatory response. *Respir Physiol* **112:**123-134.

Prediger RD, Batista LC, Takahashi RN (2004). Adenosine A1 receptors modulate the anxiolytic-like effect of ethanol in the elevated plus-maze in mice. *Eur J Pharmacol* **499:**147-154.

Ramkumar V, Bumgarner JR, Jacobson KA, Stiles GL (1988). Multiple components of the A1 adenosine receptor-adenylate cyclase system are regulated in rat cerebral cortex by chronic caffeine ingestion. *J Clin Invest* **82:**242–247.

Rasmussen BB, Brix TH, Kyvik KO, Brøsen K (2002). The interindividual differences in the 3-demthylation of caffeine alias CYP1A2 is determined by both genetic and environmental factors. *Pharmacogenetics* **12:**473-478.

Reid JL, Dawson D, Macrae IM (1995). Endothelin, cerebral ischaemia and infarction. *Clin Exp Hyperten* **17:**399-407.

Ribeiro JA, Sebastião AM (2010). Caffeine and adenosine. *J Alzheimers Dis* **20:** Suppl 1:S3-15.

Ribeiro JA, Sebastião AM, de Mendonça A (2002). Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol* **68:**377-392.

Rivkees SA, Wendler CC (2011). Adverse and protective influences of adenosine on the newborn and embryo: implications for preterm white matter injury and embryo protection. *Pediatr Res* **69:**271-278.

Rose JB, Naydenova Z, Bang A, Eguchi M, Sweeney G, Choi DS, Hammond JR, Coe IR (2010). Equilibrative nucleoside transporter 1 plays an essential role in cardioprotection. *Am J Physiol Heart Circ Physiol* **298:**H771-777.

Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB (1992). Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol Sci.* **13:**439-445.

Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, Jacobson MA (2000). Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J Biol Chem* **275:**4429–4434.

Sattin A, Rall TW (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3, 5-phosphate content of guinea pig cerebral cortex slices. *Mol Pharmacol* **6:**13–23.

Schmidt B, Roberts RS, Davis P, Doyle LW, Barrington KJ, Ohlsson A, Solimano A, Tin W (2007). Caffeine for Apnea of Prematurity Trial Group. Long-term effects of caffeine therapy for apnea of prematurity. *N Engl J Med* **357:**1893-1902.

Schulte G, Fredholm BB (2003). Signalling from adenosine receptors to mitogen-activated

protein kinases. *Cell Signal* **15:**813-827.

Sebastião AM, Ribeiro JA (2009). Adenosine receptors and the central nervous system. *Handb Exp Pharmacol* **193:**471-534.

Shahjouei S, Cai PY, Ansari S, Sharififar S, Azari H, Ganji S, Zand R (2016). Middle Cerebral Artery Occlusion Model of Stroke in Rodents: A Step-by-Step Approach. *J Vasc Interv Neurol* **8:**1-8.

Shen Q, Duong TQ (2016). Magnetic Resonance Imaging of Cerebral Blood Flow in Animal Stroke Models. *Brain Circ* **2:**20-27.

Shi D, Nikodijevic O, Jacobson KA, Daly JW (1994). Effects of chronic caffeine on adenosine, dopamine and acetylcholine systems in mice. *Arch Int Pharmacodyn Ther* **328:**261-287.

Shinohara M, Saitoh M, Nishizawa D, Ikeda K, Hirose S, Takanashi J, Takita J, Kikuchi K, Kubota M, Yamanaka G, Shiihara T, Kumakura A, Kikuchi M, Toyoshima M, Goto T, Yamanouchi H, Mizuguchi M (2013). ADORA2A polymorphism predisposes children to encephalopathy with febrile status epilepticus. *Neurology* **80:**1571-1576.

Siesjo BK (1992). Pathophysiology and treatment of focal cerebral ischemia. Part I: Pathophysiology. *J Neurosurg* **77:**169-184.

Signorello LB, Nordmark A, Granath F, Blot WJ, McLaughlin JK, Annerén G, Lundgren S, Ekbom A, Rane A, Cnattingius S. (2001). Caffeine metabolism and the risk of spontaneous abortion of normal karyotype fetuses. *Obstet Gynecol* **98:**1059-1066.

Silva CG, Porciúncula LO, Canas PM, Oliveira CR, Cunha RA (2007). Blockade of adenosine A(2A) receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. *Neurobiol Dis* **27:**182-189.

Soylu H, Zhang D, Buist R, Martin M, Albensi BC, Parkinson FE (2012). Intracortical injection

of endothelin-1 induces cortical infarcts in mice: effect of neuronal expression of an adenosine transporter. *Exp Transl Stroke Med* **4:**4.

Sozmen EG, Kolekar A, Havton LA, Carmichael ST (2009). A white matter stroke model in the mouse: axonal damage, progenitor responses and MRI correlates. *J Neurosci Meth* **180:**261-272.

Spiegler M, Villapol S, Biran V, Goyenvalle C, Mariani J, Renolleau S, Charriaut-Marlanque C (2007). Bilateral changes after neonatal ischemia in the P7 rat brain. *J Neuropathol Exp Neurol* **66:**481-490.

Stevenson DK (2007). On the caffeination of prematurity. *N Engl J Med* **357:**1967-1968.

Stockwell J, Chen Z, Niazi M, Nosib S, Cayabyab FS (2016). Protein phosphatase role in adenosine A1 receptor-induced AMPA receptor trafficking and rat hippocampal neuronal damage in hypoxia/reperfusion injury. *Neuropharmacology* **102:**254-265.

Stone TW, Ceruti S, Abbracchio MP (2009). Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. *Handb Exp Pharmacol* **193:**535-587.

Sullivan FM (1988). Reproductive toxicity tests: retrospect and prospect. *Hum Toxicol* **7:**423-427.

Svenningsson P, Nomikos GG, Fredholm BB (1999). The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. *J Neurosci* **19:**4011-4022.

Szydlowska K, Tymianski M (2010). Calcium, ischemia and excitotoxicity. *Cell Calcium* **47:**122-129.

Thauerer B, Zur Nedden S, Baier-Bitterlich G (2012). Purine nucleosides: endogenous neuroprotectants in hypoxic brain. *J Neurochem* **121:**329-342.

Tofovic SP, Kost CK Jr, Jackson EK, Bastacky SI (2002). Long-term caffeine consumption exacerbates renal failure in obese, diabetic, ZSF1 (fa-fa(cp)) rats. *Kidney International* **61:**1433-1444.

Tolstrup JS, Kjaer SK, Munk C, Madsen LB, Ottesen B, Bergholt T, Grønbaek M (2003). Does caffeine and alcohol intake before pregnancy predict the occurrence of spontaneous abortion? *Hum Reprod* **18:**2704-2710.

Trincavelli ML, Marroni M, Tuscano D, Ceruti S, Mazzola A, Mitro N, Abbracchio MP, Martini C (2004). Regulation of A2B adenosine receptor functioning by tumour necrosis factor a in human astroglial cells. *J Neurochem* **91:**1180–1190.

Tye K, Pollard I, Karlsson L, Scheibner V, Tye G (1993). Caffeine exposure in utero increases the incidence of apnea in adult rats. *Reprod Toxicol* **7:**449-452.

van den Tweel ER, Kavelaars A, Lombardi MS, Nijboer CH, Groenendaal F, van Bel F, Heijnen CJ (2006). Bilateral molecular changes in a neonatal rat model of unilateral hypoxic-ischemic brain damage. *Pediatr Res* **59:**434-439.

van Calker D, Müller M, Hamprecht B (1978). Adenosine inhibits the accumulation of cyclic AMP in cultured brain cells. *Nature* **276:**839-841.

Varma MR, Dixon CE, Jackson EK, Peters GW, Melick JA, Griffith RP, Vagni VA, Clark RS, Jenkins LW, Kochanek PM (2002). Administration of adenosine receptor agonists or antagonists after controlled cortical impact in mice: effects on function and histopathology. *Brain Res* **951:**191-201.

Verhaeghe RH, Vanhoutte PM, Shepherd JT (1977). Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenine nucleotides. *Circ Res* **40:**208-215.

Villapol S, Bonnin P, Fau S, Baud O, Renolleau S, Charriaut-Marlangue C (2009). Unilateral

blood flow decrease induces bilateral and symmetric responses in the immature brain. *Am J Pathol* **175:**2111-2120.

von Lubitz DK (1999). Adenosine and cerebral ischemia: therapeutic future or death of a brave concept? *Eur J Pharmacol* **371:**85-102.

von Lubitz DK (2001). Adenosine in the treatment of stroke: yes, maybe, or absolutely not? *Expert Opin Investig Drugs* **10:**619-632.

Wall MJ, Dale N (2007). Auto-inhibition of rat parallel fibre-Purkinje cell synapses by activity-dependent adenosine release. *J Physiol* **581:**553-565.

Wall MJ, Dale N (2008). Activity-dependent release of adenosine: a critical re- evaluation of mechanism. *Curr Neuropharmacol* **6:**329-337.

Wang Y, Cheung PT, Shen GX, Bhatia I, Wu EX, Qiu D, Khong PL (2007). Comparing diffusion-weighted and T2-weighted MR imaging for the quantification of infarct size in a neonatal rat hypoxic-ischemic model at 24h post-injury. *Int J Dev Neurosci* **25:**1-5.

Wang Y, Jin K, Greenberg DA (2007). Neurogenesis associated with endothelin- induced cortical infarction in the mouse. *Brain Res* **1167:**118-122.

Watt AH, Reid PG, Stephens MR, Routledge PA (1987). Adenosine-induced respiratory stimulation in man depends on site of infusion. Evidence for an action on the carotid body? *Br J Clin Pharmacol* **23:** 486–490.

Weaver DR (1993). A2a adenosine receptor gene expression in developing rat brain. *Brain Res Dev Brain Res* **20**, 313-327.

Weaver DR (1996). A1-adenosine receptor gene expression in fetal rat brain. *Brain Res Dev Brain Res* **20**, 205-23.

Wegener S, Weber R, Ramos-Cabrer P, Uhlenkueken U, Sprenger C, Wiedermann D, Villringer

A, Hoehn M (2006). Temporal profile of T2-weighted MRI distinguishes between pannecrosis and selective neuronal death after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab* **26:**38-47.

Wilkinson JM, Pollard I (1993). Accumulation of theophylline, theobromine and paraxanthine in the fetal rat brain following a single oral dose of caffeine. *Brain Res Dev Brain Res* **75:**193-199.

Williams-Karnesky RL, Stenzel-Poore MP (2009). Adenosine and stroke: maximizing the therapeutic potential of adenosine as a prophylactic and acute neuroprotectant. *Curr Neuropharmacol* **7:**217-227.

Windle V, Szymanska A, Granter-Button S, White C, Buist R, Peeling J, Corbett D (2006). An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. *Exp Neurol* **201:**324-34.

Wisborg K, Kesmodel U, Bech BH, Hedegaard M, Henriksen TB (2003). Maternal consumption of coffee during pregnancy and stillbirth and infant death in first year of life: prospective study. *BMJ* **326:**420.

Xu Z, Xu P, Chen Y, Liu J, Zhang Y, Lv Y, Luo J, Fang M, Zhang J, Wang J, Wang K, Wang X, Chen G (2015). ENT1 inhibition attenuates epileptic seizure severity via regulation of glutamatergic neurotransmission. *Neuromolecular Med* **17:**1-11.

Yang A, Palmer AA, de Wit H (2010). Genetics of caffeine consumption and responses to caffeine. *Psychopharmacology* **211:**245-257.

Yoder B., Thomson M, Coalson J (2005). Lung function in immature baboons with respiratory distress syndrome receiving early caffeine therapy: a pilot study. *Acta Paediatr* **94:** 92–98.

Young JD, Yao SY, Sun L, Cass CE, Baldwin SA (2008). Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins. *Xenobiotica* **38:**995-1021.

Yu QJ, Tao H, Wang X, Li MC (2015). Targeting brain microvascular endothelial cells: a therapeutic approach to neuroprotection against stroke. *Neural Regen Res* **10:**1882-1891.

Zaidi SI, Jafri A, Martin RJ, Haxhiu MA (2006). Adenosine A2A receptors are expressed by GABAergic neurons of medulla oblongata in developing rat. *Brain Res* **1071:**42-53.

Zamzow CR, Xiong W, Parkinson FE (2008a). Astrocytes affect the profile of purines released from cultured cortical neurons. *J Neurosci Res* **86**:2641-2649.

Zamzow CR, Xiong W, Parkinson FE (2008b). Adenosine produced by neurons is metabolized to hypoxanthine by astrocytes. *J Neurosci Res* **86:**3447-3455.

Zhang D, Xiong W, Albensi BC, Parkinson FE (2011). Expression of human equilibrative nucleoside transporter 1 in mouse neurons regulates adenosine levels in physiological and hypoxic-ischemic conditions. *J Neurochem* **118:**4-11.

Zhao J, Gonzalez F, Mu D (2011). Apnea of prematurity: from cause to treatment. *Eur J Pediatr* **170:**1097-1105.