Behavioural Effects of Enhanced Expression of Equilibrative Nucleoside Transporter 1 or Knockout of Ecto-5'-Nucleotidase in Mice

by

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ABSTRACT

Adenosine is an important neuromodulator. In the present study, we used mice with expression of human ENT1 (hENT1) and deficiency of CD73, respectively, to address the relative importance of intracellular and extracellular pathways in adenosine regulation. [^{3}H]Nitrobenzylthioinosine binding assays were performed and found increased expression of hENT1 with increased gene dose. We performed a series of behavioural experiments with caffeine and ethanol and compared hENT1 heterozygous and homozygous transgenic mice to their wild type littermates. We found that the expression of hENT1 leads to a change in behavioural responses relative to wild type mice, but no sign of a gene dose dependent increase was observed. With CD73 knockout mice, we performed a series of behavioural experiments with caffeine and ethanol that showed a change in adenosine related behaviours. We also performed experiments that tested anxiety-like behaviours and found reduced anxiety-like behaviours with CD73 knockout mice relative to wild type mice. These studies show that mice with enhanced expression of ENT1 or knockout of CD73 have altered extracellular level of adenosine.

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ABBREVIATIONS

A_1R – adenosine 1 receptors

$A_{2A}R$ – adenosine 2A receptors

$A_{2B}R$ – adenosine 2B receptors

A_3R – adenosine 3 receptors

AC – adenylate cyclase

ADA – adenosine deaminase

ADP – adenosine diphosphate

ADO – adenosine

AK – adenosine kinase

AMP – adenosine deaminase

AR – adenosine receptors

ATP – adenosine triphosphate

bp – base pairs

BSA – bovine serum albumin

cAMP – cyclic adenosine monophosphate

C1-IB-MECA – 2-chloro-N6-(3-iodobenzyl) adenosine-5′-N-methyluronamide

CD73 – ecto-5'-nucleotidase

CGS 21680 – 2-[p-(2-carbonylethyl)phenylethylamino]-50-N-ethylcarboxamidoadenosine

CHA – N6-cyclohexyladenosine

CNS – central nervous system

CNT – concentrative nucleoside transporter

COPD - chronic obstructive pulmonary disease

CPA – N^6-cyclopentyladenosine

CCPA - 2-chloro-N6-cyclopentyladenosine

CREB – cAMP response element binding protein

DAG – diacylglycerol

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

dNTPs – deoxynucleoside triphosphates

DPCPX – 1,3-dipropyl-8-cyclophentylxanthine

EDTA – ethylenediaminetetraacetic acid

EPM – elevated plus maze

ETM – elevated T maze

G_i / G_o – inhibitory G protein

G_{olf} – olfactory G protein

G_s – stimulatory G protein

GABA – γ-amino butyric acid

GI - gastrointestinal

GMP – guanosine monophosphate

GPCRs – G protein coupled receptors

hENT – human equilibrative nucleoside transporter

IPC – ischemic preconditioning

kb – kilo base pairs

KO – knockout

LORR – loss of righting reflex

MAPK – mitogen-activated protein kinase

MRS1220 – N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene

MRS1754 – [N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide]

NBMPR – nitrobenzylthiolinosine

NMDA – N-methyl-D-aspartate

PCR – polymerase chain reaction

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase-C

SAH – S-adenosylhomocysteine

SLC – Solute Carrier

ZM 241385 – 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-yl-amino]ethyl]phenol

Chapter 1. INTRODUCTION

Adenosine (ADO) is the endogenous purine nucleoside base of adenosine triphosphate (ATP). It is an important molecule that was first described in 1929 for its potent action on mammalian heart (Drury *et al.*, 1929). Since then, the metabolic pathways that result in adenosine formation have been widely studied. Adenosine is produced in the extracellular space by the breakdown of ATP involving a series of enzymes including ecto-5'-nucleotidase (CD73). Adenosine is released from almost all cells. Also, many of the physiological effects of adenosine have been described. Adenosine has diverse physiological effects through the body by activating its receptors on the cell surface. Adenosine was found to have effects such as vasodilation (Collis, 1989), decreased cardiac rate and contractility (Berne, 1963; Drury *et al.*, 1929), sedation (Phillis *et al.*, 1982), inhibition of platelet aggregation (Ledent *et al.*, 1997), inhibition of lipolysis (Fredholm *et al.*, 2001), regulation of gluconeogenesis (Lavoinne *et al.*, 1987), and prevention or induction of apoptosis (Vitolo *et al.*, 1998; Wen *et al.*, 2003). As a neuromodulator, adenosine has effects on neuronal excitability, locomotor activity and arousal (Parkinson *et al.*, 2009). The regulation of adenosine has been investigated as a therapeutic strategy for diseases including: ischemia (Deckert *et al.*, 1994; Fredholm, 1997; Lasley *et al.*, 1990; Rudolphi *et al.*, 1992), epilepsy (Wiesner *et al.*, 1999; Zhang *et al.*, 1993), Huntington's disease (Glass *et al.*, 2000; Reggio *et al.*, 1999), schizophrenia (Boison *et al.*, 2012; Boszormenyi-Nagy *et al.*, 1956; Ferre, 1997; Kafka *et al.*, 1996; Tsai, 2005), Parkinson's disease (Fuxe *et al.*, 1992; Richardson *et al.*, 1999; Simola *et al.*, 2006), asthma (Auchampach *et al.*, 1997; Feoktistov *et al.*, 1995; Hayashida *et al.*, 2005; Mahmoud *et al.*,

2011; Mauborgne *et al.*, 2002; Sollevi, 1997), pain control (Hayashida *et al.*, 2005; Mahmoud *et al.*, 2011; Mauborgne *et al.*, 2002; Sollevi, 1997) and inflammation (Cronstein, 1994). Adenosine is still rarely used in therapy, except for the treatment of cardiac arrhythmias. Clinical trials are ongoing for the therapeutic potential of regulating adenosine signaling in inflammation, schizophrenia, heart failure, Parkinson's disease and neuropathic pain (Fredholm *et al.*, 2011).

1.1 Physiological roles of adenosine

Extracellular basal concentrations of adenosine span a wide range, with the most estimated levels of adenosine in the range of 25-250 nM (Dunwiddie *et al.*, 2001). Extracellular adenosine can rise to micromolar levels in conditions of ischemic events (Fredholm *et al.*, 2005). Pathophysiological stimuli such as hypoxia (Frenguelli *et al.*, 2003), and ischemia (Parkinson *et al.*, 2000) can lead to the huge increase of extracellular adenosine. ATP is an energy source used for most cellular activity. Adenosine can be produced from ATP intracellularly. Adenosine has been named as a 'retaliatory metabolite' (Newby, 1984) because of its ability to reset energy balance. When energy supply cannot meet energy demand, adenosine is formed to decrease the demand of energy and increase energy supply (vasodilation) (Mubagwa *et al.*, 2001).

The exploration of adenosine as a signaling molecule has been ongoing since the study of Szent-Gyorgy for the effect of purines effect on the heart (Drury *et al.*, 1929). After that, the physiological and pharmacological functions of adenosine have been widely studied. Adenosine is recognized to be involved in many physiological events. After decades of studies,

adenosine has been found to play important roles in cardiovascular system and regulating the blood flow to tissues including heart, liver and brain (Berne, 1980; Latini *et al.*, 2001), as well as modulating lipolysis, immune response and gastrointestinal function (Fredholm *et al.*, 2001). Adenosine slows atrioventricular conduction and sinus rhythm (Tai *et al.*, 1990); it can be used for supraventricular tachycardia because of its bradycardic effect (DiMarco, 1987). Adenosine administered in an aerosol induces a bronchoconstriction in asthmatic patients (Lasley *et al.*, 1990; Ng *et al.*, 1990). During post-ischemic reperfusion of heart, the damage is reduced because of its anti-inflammatory and anti-thrombotic effects (Jordan *et al.*, 1997; Lasley *et al.*, 1990; Rudolphi *et al.*, 1992). Adenosine inhibits platelet aggregation and also has been shown to modulate immune reactions (Ledent *et al.*, 1997). As well, adenosine inhibits intestinal secretion (Hancock *et al.*, 1995) and regulates the release of renin in the renal system (Jackson, 1991; Virdis *et al.*, 1999).

Beyond the peripheral effects, adenosine also takes great part in the modulation of the central nervous system (CNS). Adenosine is a neuromodulator, which modulates neurotransmitter release and neuronal excitability. It has complex effects, in that it can reduce or increase release of neurotransmitters such as glutamate and norepinephrine (Latini *et al.*, 2001). Adenosine may have inhibitory effects on motor stimulation to some extent. Motor activity can be stimulated by the methylxanthine caffeine, which is a non-selective adenosine receptor antagonist. Adenosine may also take part in the regulation of pain nociception (Sweeney *et al.*, 1991; Sweeney *et al.*, 1989). Agonists of adenosine receptors that are delivered into the brain have anticonvulsant effects (Wiesner *et al.*, 1999; Zhang *et al.*, 1993). Adenosine has also been found to play a role in regulation of sleep-wake cycles (Chagoya de Sanchez *et al.*, 1993). Adenosine may be critical in the promotion of sleep (Huang *et al.*,

2011). Also, adenosine has sedative effects and evidence suggests a direct involvement of adenosine in regulation of anxiety (Florio *et al.*, 1998; Ledent *et al.*, 1997; Maximino *et al.*, 2011). Finally, adenosine plays an important role in protection against brain injuries caused by ischemic and excitotoxic events (Schubert *et al.*, 1994) as mentioned above.

1.2 Adenosine pathways in physiology and pathology, including intracellular enzymes

Adenosine is a homeostatic modulator and neuromodulator (Cunha, 2001). Adenosine is neither stored in vesicles nor released by exocytosis. Adenosine binds to and activates its four G protein coupled receptors on cell surfaces. In that case, adenosine has to be present in the extracellular milieu in order to work. Adenosine can be formed intracellularly and released through different nucleoside transporters. Increased levels of adenosine inside the cell produce a concentration gradient across the cell membrane. Equilibrative nucleoside transporters (ENTs) transport adenosine across cell membranes in a direction determined by the concentration gradient of adenosine. In contrast, concentrative nucleoside transporters (CNTs) utilize the sodium gradient and transport adenosine plus sodium into cells. Adenosine can also be formed extracellularly through the ecto-nucleotidase pathway from ATP, or other adenine nucleotides released from the cell and signaling through purinergic receptor families P2X and P2Y. Moreover, adenosine can be formed extracellularly after the release of cAMP and its hydrolysis by extracellular phosphodiesterases (Dunwiddie *et al.*, 2001; Latini *et al.*, 2001).

Intracellularly, adenosine is mostly produced from AMP, but also maybe formed from S-adenosylhomocysteine (SAH) hydrolase (Dunwiddie *et al.*, 2001). Under conditions where energy supply cannot meet energy requirement, ATP is hydrolyzed to ADP and AMP, AMP is then catalyzed by the enzyme 5'-nucleotidase and form adenosine. Therefore, levels of

adenosine rise dramatically inside the cell. Through a bidirectional ENT, extracellular concentrations of adenosine will also increase in order to keep the parallel concentration levels on both sides of the cell (Fredholm *et al.*, 2005).

The increase in extracellular adenosine levels is transient. Clearance of extracellular adenosine is mostly by uptake into cells through the action of bidirectional ENTs followed by its metabolism inside cells. Intracellularly, adenosine can be converted to inosine by an irreversible deamination catalyzed by adenosine deaminase (ADA). Also, adenosine can be converted back to AMP through the action of adenosine kinase (AK). The AK pathway is more favorable at lower adenosine concentrations whereas ADA pathway is preferred at high adenosine concentration. AK requires a phosphate from ATP so this pathway is reduced when ATP levels are low. The affinity value of AK to adenosine is one or two orders of magnitude lower than ADA (Phillips *et al.*, 1979). AK is saturated at physiological concentration. When adenosine concentration rises, AK activity is inhibited. Adenosine may also be cleared by extracellular deamination catalyzed by ecto-adenosine deaminase, thus adenosine will be converted into its metabolite inosine (Franco *et al.*, 1997). However, to what extend this pathway works for adenosine clearance in CNS remains to be established (Cunha *et al.*, 2001).

Another pathway that may also be relevant for the intracellular metabolism of adenosine is the SAH pathway. A reversible reaction of SAH hydrolase catalyzes SAH to form adenosine. This pathway only represents a minor source of adenosine, because its levels in the brain are very low (Reddington *et al.*, 1983).

The actual concentration of extracellular adenosine is difficult to measure due to the transient rise of the adenosine level and its clearance. In addition, any cell injury can cause increases in adenosine levels, so measured levels are artificially high because of the methods

used to measure it. Pharmacological manipulations of the critical enzymes and transporters in the adenosine pathway can be used to further investigate the exact site of adenosine formation and the mechanisms of adenosine removal.

1.2.1 Nucleoside transporters

Nucleoside transporters are important for the cellular uptake and the release of adenosine. Therefore, nucleoside transporters play a critical role in controlling the levels of extracellular adenosine. There are two different families of nucleoside transporters that have been identified in mammals: concentrative nucleoside transporters (CNT), also known as Solute Carrier (SLC) 28, and equilibrative nucleoside transporters (ENT), also known as SLC29. In the CNS, ENTs appear to be dominant (Thorn *et al.*, 1996).

1.2.1.1 Equilibrative nucleoside transporters

The ENTs are a family of transporters, which transport nucleosides across cell membranes from the location with the higher concentration to the region with the lower concentration. Net transport activity ceases when the concentration gradient dissipates. Four subtypes of ENT have been identified: ENT1, ENT2, ENT3, and ENT4. Among these subtypes, ENT1 and ENT2 are best characterized. Based on their sensitivity to inhibition by nitrobenzylthioinosine (nitrobenzylmercaptopurine riboside or NBMPR), they have also been classified as NBMPR sensitive (ENT1) or insensitive (ENT2) (Baldwin *et al.*, 2004).

The ENT1 transporter can be inhibited by NBMPR at nanomolar levels (Pickard *et al.*, 1973), which is different from ENT2. Therefore, [^{3}H]NBMPR is widely used as a selective radioligand for the study of ENT1. ENT1 has been found to be important in the regulation of

adenosine levels. According to results with ENT1 knockout mice in several studies (Chen *et al.*, 2007; Choi *et al.*, 2004), mice without ENT1 had a reduced adenosine receptor signaling in the brain. The ENT1 knockout mice have shown increased exploratory behaviour, and also reduced response to ethanol. However, the actually role that ENT1 plays in adenosine regulation is still not yet clear.

Previously, our lab generated mice with neuronal expression of human ENT1 (hENT1) on CD1 background and found that heterozygous hENT1 transgenic mice showed increased expression and function in brain compared to their wild type littermates. The study indicated hENT1 transgenic mice significantly increased [^{3}H]NBMPR binding and increased [^{3}H]adenosine uptake in cortical tissues relative to wild type mice. Behavioural response to caffeine and ethanol (Parkinson *et al.*, 2009), two drugs that act directly or indirectly through adenosine receptors, have been tested. The results have shown a reduced sensitivity to the locomotor stimulation effect of caffeine, and an increased hypnotic effect of ethanol, relative to wild type mice.

The ENT2 transporter is distinguished from ENT1 because of its lower sensitivity to inhibition by NBMPR; micromolar concentrations are required for the inhibition of ENT2 (Cass *et al.*, 1998). ENT2 is more abundant in rat than mouse or human. In human brain, the highest expression of ENT2 is in the cerebellum and brainstem. Relatively lower expression of ENT2 is expressed in cerebral cortex, basal ganglia, and hippocampus (Jennings *et al.*, 2001). ENT2 also transports a wide range of pyrimidine and purine nucleosides. ENT2 has a lower apparent affinity to all nucleosides compared to ENT1, except for inosine (Crawford *et al.*, 1998; Griffiths *et al.*, 1997; Ward *et al.*, 2000).

The ENT3 and ENT4 may be proton-dependent transporters, but have not yet been well

characterized. Multiple tissue RNA analysis suggested that hENT3 was broadly expressed in human tissues with particular abundance in placenta. ENT4 is also likely to be ubiquitously distributed in human tissues (Baldwin *et al.*, 2004).

1.2.1.2 Concentrative nucleoside transporters

The CNTs are coded for by the SLC28 gene family. Nucleosides are transported through these transporters against their concentration gradient in a sodium-dependent manner. Three members in this family have been cloned and characterized in the past decades: CNT1, CNT2 (or sodium-dependent purine nucleoside transporter, SPNT), and CNT3. These subtypes have different substrate specificities (Gray *et al.*, 2004).

CNT1 is found mainly in liver, kidney, and small intestine (epithelia apical membrane). It is a pyrimidine nucleoside preferring transporter. In addition, it transports adenosine in a low-capacity, high-affinity manner (Gray *et al.*, 2004).

CNT2 has been found in human tissues including the liver, kidney, heart, brain, placenta, skeletal muscle, colon, pancreas, rectum, duodenum, ileum, and jejunum through Northern blot analysis (Gray *et al.*, 2004). CNT2 predominantly transports purine nucleosides, including guanosine, formycin B and adenosine. Unless the sodium gradient is disturbed, CNT2 transports purines into the cell (Borgland *et al.*, 1997).

CNT3 is the third sodium-dependent nucleoside transporter subtype that has been cloned (Ritzel *et al.*, 2001). CNT3 transport both purines and pyrimidines. More studies are required on this transporter (Gray *et al.*, 2004).

Three other sodium-dependent nucleoside transporters are known but not well characterized. Further studies are needed to establish their roles.

1.2.2 Ecto-5'-nucleotidase (CD73) pathway

ATP is an important source of extracellular adenosine. ATP can appear in the extracellular environment through different pathways. First, vesicular release of ATP from the synaptic vesicles occurs on the stimulation of nerve terminals (Cunha, 2001). Second, ATP can be released through non-exocytotic mechanisms. All cell types in the CNS may contribute to the release of ATP (Caciagli *et al.*, 1988; Queiroz *et al.*, 1997). Studies suggests that synaptic activity leads to vesicular release of ATP (Pankratov *et al.*, 2006), and the rise of ATP levels leads to a rise of Ca^{2+} levels in neighboring astrocytes. The elevated Ca^{2+} levels lead to a cellular efflux of ATP from astrocytes (Coco *et al.*, 2003). Adenosine can be produced extracellularly from ATP via a series of steps involving enzymes including ecto-apyrases (CD39) and ecto-5'-nucleotidase (CD73) (Zimmermann, 2000). CD73 is critical because the dephosphorylation of AMP by CD73 is the final step of extracellular adenosine formation (Yegutkin, 2008).

Studies showed that CD73 has many physiology roles, such as immune regulation, protection against bleomycin-induced lung injury and ventilator-induced acute lung injury, prevention of vascular leak and neutrophil infiltration in hypoxia, and protection against renal and myocardial ischemia (Hasko *et al.*, 2011). Recently our laboratory has used CD73 knockout mice obtained from Dr. Linda Thompson (Thompson *et al.*, 2004) as a new tool to study the regulation of adenosine concentration. By knocking out CD73 on the cell surface, the formation of extracellular adenosine is expected to be reduced. Studies using CD73 knockout mice have shown altered physiological responses in these animals. The study that investigated the regulation of tubuloglomerular feedback of glomerular filtration rate in CD73

knockout mice suggested that, the generation of adenosine at the glomerular pole depends on the activity of CD73 (Castrop *et al.*, 2004). In the study of Thompson, et al. CD73 knockout mice showed a significantly accentuation of vascular leakage in multiple tissues including lung, liver, and skeletal muscles during hypoxia (Thompson *et al.*, 2004). CD73 knockout mice also showed alteration of thromboregulation and augmentation of vascular inflammatory response, suggesting an important physiological role of (Koszalka *et al.*, 2004). Information is absent concerning a behavioural role of CD73 knockout. One of the aims of this thesis will be to assess the effects of CD73 knockout on behavior in these mice.

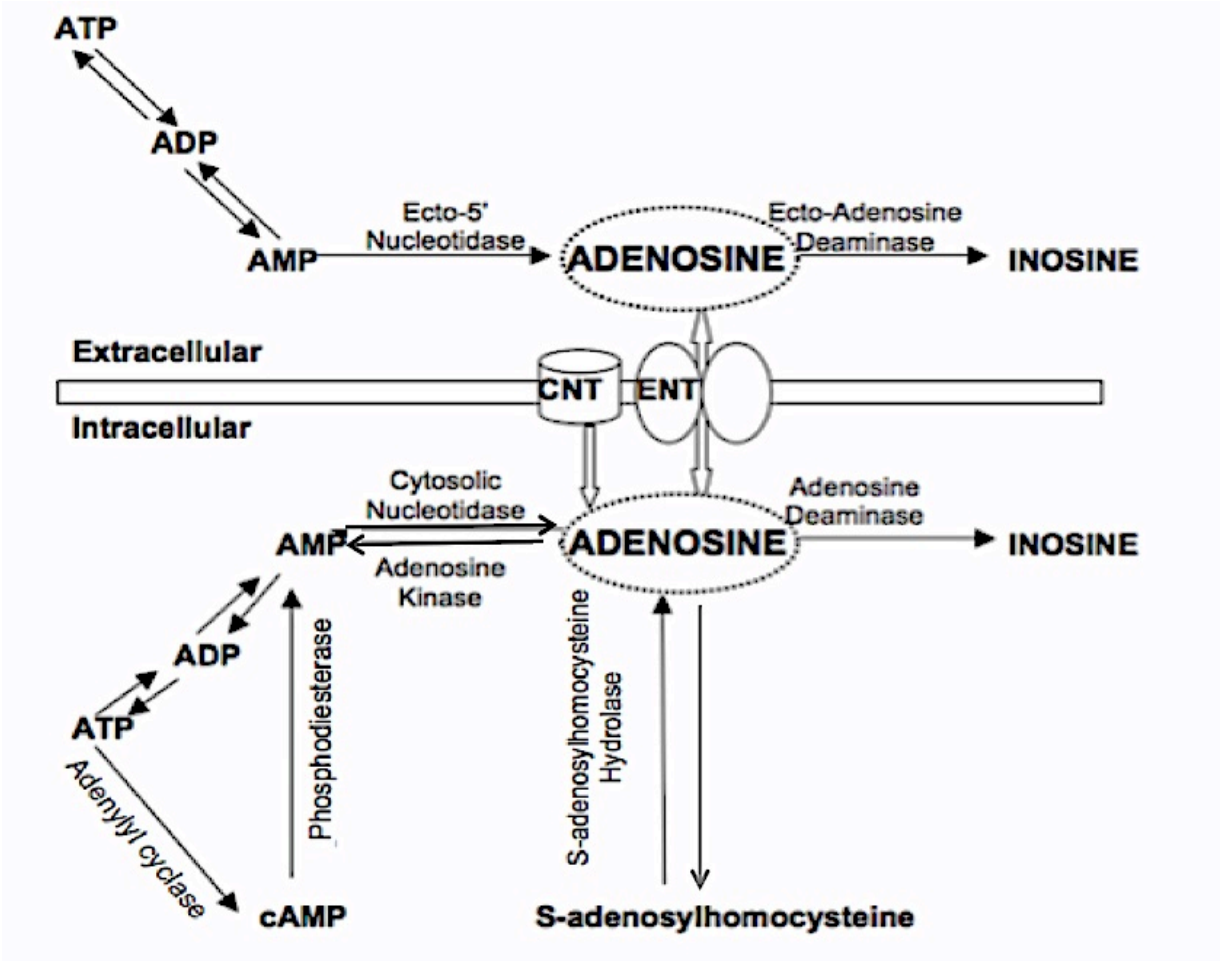


Figure 1. Schematic Diagram Of Adenosine Production, Transport, And Metabolism.

Adenosine can be produced extracellularly from ATP catalyzed by ecto-5' nucleotidase. Also, adenosine can be formed intracellularly from ATP dephosphorylation. cAMP can be metabolized to AMP by phosphodiesterase, which also provides a source of adenosine. Adenosine can be transported through cell membranes via nucleoside transporters (CNTs, ENTs). Adenosine can be metabolized by adenosine deaminase to produce inosine, or phosphorylated by adenosine kinase to produce AMP.

1.3 Adenosine receptors

Adenosine receptors (ARs) were defined in the 1970s (Fredholm *et al.*, 1994). Adenosine produces its physiological effects through the activation of four G-protein coupled receptors (GPCRs): Adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. Among these four receptors, adenosine A_1 and A_{2A} receptors are well characterized, whereas less is known about the A_{2B} and A_3 receptors.

1.3.1 Adenosine A_1 receptor

The adenosine A_1 receptor (A_1R) was first cloned from canine tissue in 1989 (Libert *et al.*, 1989). Since then, A_1Rs for several animal species have been cloned. The sequence is characterized by a high similarity across species including human (Libert *et al.*, 1992; Townsend-Nicholson *et al.*, 1992). A_1R is coupled to members of inhibitory G proteins (G_i/G_o). When adenosine binds to A_1R, it inhibits the activity of adenylate cyclase (AC). Decreased activity of AC can lead to decreased cellular levels of cyclic adenosine monophosphate (cAMP) (Fredholm, 1995b). Moreover, phospholipase-C (PLC)-β can be activated. The activation of PLC-β can involve two different mechanisms: activation via released G_i betagamma subunits (Dickenson *et al.*, 1998; Gerwins *et al.*, 1992), and the coupling of A_1R to a G_q alpha subunit (Cordeaux *et al.*, 2004). Inhibition of neurotransmitter release is another important result of adenosine signaling at A_1R. Glutamate, acetylcholine, norepinephrine, dopamine, and serotonin release can be reduced by the activation of A_1R (Corradetti *et al.*, 1984a; Corradetti *et al.*, 1984b; Dolphin *et al.*, 1983; Prince *et al.*, 1992).

A_1Rs are found in all cell types in the brain: astrocytes, microglia, oligodendrocytes, and neurons (Fredholm *et al.*, 2001). For studying A_1R, the agonist N^6-cyclopentyladenosine (CPA)

and antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) have been useful. The radioligands [^{3}H]DPCPX and [^{3}H]CPA have been used as probes for receptor binding assays (Fredholm *et al.*, 2001).

Adenosine A_1 receptors have a role in many physiological functions. A_1R activation can mediate inhibition of neurotransmitter release at the presynaptic level and it also induces neuronal hyperpolarization at the postsynaptic level. Activation of A_1Rs may be useful for the anticonvulsant, anxiolytic, sedative, and locomotor depression effects of adenosine (Fredholm *et al.*, 2005). Adenosine has an important role in pain modulation. Activation of A_1R can increase K^+ conductance and inhibit presynaptic sensory nerve terminals, decrease the release of glutamate and substance P, thus inhibit the intrinsic neurons and produce antinociceptive effects. A_1Rs have effects in the CNS as well as peripheral tissues. A_1R has been shown to have a protective and anti-inflammatory role in asthma and chronic obstructive pulmonary disease (Gessi *et al.*, 2011). At the cardiovascular level, A_1R mediate bradycardia, inotropic, and dromotropic effects. Adenosine has an ischemic preconditioning effect that protects myocardium from infarction by a subsequent prolonged ischemic insult (Fredholm *et al.*, 2005). In the kidney, A_1R facilitates vasoconstriction, inhibition of glomerular filtration rate, renin secretion, neurotransmitter release, and tubuloglomerular feedback. Several studies suggest A_1R activation is protective by inhibiting inflammation, necrosis, and apoptosis (Fredholm *et al.*, 2005).

Knockout-mice are useful tools for studying the function of receptors. A_1Rs are largely expressed in different brain regions such as the cerebral cortex, hippocampus, and cerebellum (Fredholm *et al.*, 2005). A_1R knockout mice have been observed to have an increased anxiety-related behaviour compared to wild-type mice (Lang *et al.*, 2003). Also, knockout mice have

an alteration in nociception and have decreased tolerance to hypoxia (Johansson *et al.*, 2001). Lower survival rate of A_1R knockout mice compared with wild-type mice suggest that A_1R has played a very important role in normal physiology (Fredholm *et al.*, 2005).

1.3.2 Adenosine A_{2A} receptor

The first clone of adenosine A_{2A} receptor ($A_{2A}R$) was from canine tissues, as was A_1R (Maenhaut *et al.*, 1990). Since then, $A_{2A}Rs$ from many other species have been cloned, including human, rat, and mouse (Fredholm *et al.*, 2005). $A_{2A}R$ is coupled with the stimulatory G protein (G_s). When adenosine binds to $A_{2A}R$, activity of AC will be stimulated and lead to an increase of cAMP. In addition to G_s, presynaptic $A_{2A}R$ may also couple to G_q, causing PKC activation (Gubitz *et al.*, 1996; Lopes *et al.*, 2002; Lopes *et al.*, 1999).

Highest expression of $A_{2A}R$ can be found in the spleen, thymus, leukocytes, platelets and striatum of brain. Several mRNA distribution studies have shown high expression in striatopallidal GABAergic (GABA: γ-amino butyric acid) neurons and olfactory bulb. (Fredholm *et al.*, 2005).

2-[p-(2-carboxyethyl)phenylethylamino]-50 ethylcarboxamidoadenosine (CGS 21680) is an agonist of $A_{2A}R$ and [^{3}H]CGS 21680 is a useful radioligand for $A_{2A}R$. 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-yl-amino]ethyl]phenol (ZM 241385) is a selective antagonist of $A_{2A}R$, and radioligand [^{3}H]ZM 241385 is also a useful tool for receptor binding assays (Fredholm *et al.*, 2005). With the various distribution of adenosine $A_{2A}Rs$, $A_{2A}Rs$ have been suggested to be involved in many of the physiological functions of adenosine. $A_{2A}Rs$ in the CNS have played an important role in the regulation of sensorimotor integration in basal ganglia, and stimulation of the sensory nerve activity (Gessi *et al.*, 2011).

Also, this receptor was responsible for the inhibition of polymorphonuclear leukocytes and platelet aggregation. Activation of $A_{2A}R$ on endothelial cells, coronary smooth muscle cells, monocytes/macrophages, and foam cells can lead to angiogenesis, vasodilatation, inhibition of proinflammatory cytokine production, and inhibition of plaque formation (Fredholm *et al.*, 2005). Antagonism of $A_{2A}Rs$ reduces injury following global forebrain ischemia (Gao *et al.*, 1994). On the other hand, activation of the adenosine $A_{2A}Rs$ have been found to reduce ischemia-reperfusion injury during reperfusion of various tissues (Fredholm *et al.*, 2001).

Knockout mice have been used to identify the function of adenosine A_{2A} receptors. $A_{2A}R$ knockout mice have shown an increase in some anxiety-like behaviours in different studies, but the role of adenosine receptors in anxiety is not yet clear (Fredholm *et al.*, 2005). Also, increased aggressiveness has been observed in $A_{2A}R$ knockout mice. Adenosine A_{2A} receptors have been suggested to be involved in some neuronal disorders, including Parkinson's and Huntington's diseases (Fredholm *et al.*, 2005).

1.3.3 Adenosine A_{2B} receptor

The adenosine A_{2B} receptor ($A_{2B}R$) was first found to be distinguished from $A_{2A}R$ in 1972 according to the different sensitivities of agonists to increase cellular cAMP levels (Huang *et al.*, 1972). $A_{2B}R$ were cloned from human, rat and mouse (Baraldi *et al.*, 2008).

$A_{2B}R$ is coupled with G_s protein, like $A_{2A}R$, can lead to AC activation and increase cellular cAMP. $A_{2B}R$ has also been demonstrated to be involved in the activation of phospholipase C (PLC) through G_q protein (Pilitsis *et al.*, 1998).

Highest levels of mRNA for $A_{2B}R$ were found in the gastrointestinal (GI) tract, whereas

lower levels were found in the brain and lung (Fredholm *et al.*, 2005). The role of $A_{2B}R$ has not been as extensively studied as A_1R and $A_{2A}R$, but $A_{2B}R$ contributes to a variety of normal physiological functions. Studies have shown that activation of $A_{2B}R$ has provided a mechanism of damage control during CNS injury (Gessi *et al.*, 2011). $A_{2B}Rs$ also play an important role in the vasodilatation, cardioprotection, inhibition of macrophage and monocyte function, and mediating degranulation and activation of mast cells. $A_{2B}Rs$ also play a role in cancer development (Gessi *et al.*, 2011).

1.3.4 Adenosine A_3 receptor

Adenosine A_3 receptor (A_3R) was initially isolated from rat testis. Since then, sheep and human homologs were cloned (Gessi *et al.*, 2011).

A_3R is coupled with the inhibitory G protein (G_i). When adenosine binds to A_3R, the activity of AC can be inhibited and thus decrease the cellular levels of cAMP. In addition, through G_q protein, activation of A_3Rs activation stimulates PLC. (Gessi *et al.*, 2011).

A_3Rs play a role in a number of CNS functions including nociception, neuroprotection, locomotion, and behavioural depression(Gessi *et al.*, 2011). The A_3R may be crucial for cardioprotection in ischemia-reperfusion (Ge *et al.*, 2006).

Knockout mice have shown that the absence of A_3R may lead to decreased pain sensitivity to some stimuli. Also, A_3R knockout mice showed a slight increase in locomotor activity in the anxiety behaviour experiments, but no signs of anxiety (Fredholm *et al.*, 2005).

1.4 Life style drugs with adenosinergic mechanisms

Life style drugs, such as caffeine and ethanol, have adenosinergic mechanisms. Ethanol is a widely consumed beverage worldwide. Ethanol has its effect on the inhibition of the excitatory neurotransmitter glutamate and the facilitation of the inhibitory neurotransmitter GABA (Ikonomidou, 2000). The action of ethanol also involves adenosinergic mechanisms. Studies indicate that the hypnotic and the motor incoordination effects of ethanol are increased by adenosine receptor agonists and decreased by adenosine receptor antagonists (Dar, 1990; Dar, 2001). Adenosine A_{2A} knockout mice showed reduced hypnotic effects of ethanol (Naassila *et al.*, 2002). There are two mechanisms by which ethanol may be involved in the regulation of adenosine: 1. The metabolism of ethanol through liver can produce adenosine in a complex way. Ethanol can be metabolized to acetaldehyde by alcohol dehydrogenase; then to acetate by aldehyde dehydrogenase. The metabolism of acetate to acetyl coenzyme A consumes ATP and produces AMP. AMP can be metabolized to adenosine by the action of 5'-nucleotidases (Carmichael *et al.*, 1991). 2. Ethanol can block the ENT1 and regulation of the cellular uptake or release of adenosine. In the present study, we monitored the hypnotic effects as well as motor incoordination effects of ethanol with loss of righting reflex test and rotarod tests.

Caffeine is another lifestyle drug that works through adenosinergic mechanisms. Caffeine is a non-selective adenosine receptor antagonist. It binds and blocks all types of adenosine receptors. Caffeine has a similar affinity for A_1 and A_{2A} receptors (Fisone *et al.*, 2004). Through the blockade of adenosine receptors, caffeine is well known to stimulate locomotion in experimental animals (Waldeck, 1975) . The locomotory stimulation effect of caffeine was initially thought to be mediated by the blockade of A_1 receptor, because of the

well-known effect of A_1Rs to inhibit neuronal activity (Daly *et al.*, 1981). More recently, it was shown that inhibition of A_{2A}R contributes to the stimulatory effects of caffeine (Svenningsson *et al.*, 1997). Studies with A_{2A} knockout mice suggest that A_{2A}R blockade may be more important than A_1R antagonism for locomotor stimulation by caffeine (El Yacoubi *et al.*, 2000).

Chapter 2. OBJECTIVES AND HYPOTHESES

As described above, adenosine has important receptor mediated effects in physiological and pathological conditions. It exhibits rapid changes in concentrations during conditions such as ischemia and reperfusion. Furthermore, it plays a role in the effects of the life-style drugs caffeine and ethanol. While much is known about specific enzymes and transporters that affect the concentration of adenosine available to activate its receptors, it is still not understood how adenosine levels are regulated. The objective of this project was to characterize mice that have genetic mutations with the potential to affect adenosine levels. In the present study, mice expressing hENT1 and mice deficient in CD73 were used to address the importance of intracellular and extracellular pathways for behaviours that are influenced by adenosine receptor activity.

With these genetically modified mice, we designed different experiments to test the following hypotheses:

1. Expression of hENT1 transporters and behavioural responses involving the action of adenosine increase according to hENT1 gene dose.
2. CD73 knockout mice have decreased behavioural response involving the action of adenosine.
3. CD73 knockout mice have increased anxiety-like behaviours.

Chapter 3. MATERIALS AND METHODS

3.1 Human equilibrative nucleoside transporter 1 (hENT1) transgenic mice

Transgenic mice with neuronal expression of hENT1 were generated previously (Parkinson *et al.*, 2009) and bred at the Genetic Models Centre at the University of Manitoba. Routinely, heterozygous males were mated to wild type females to produce wild type and heterozygous littermates. For some experiments, heterozygous males were mated to heterozygous females to produce wild type, heterozygous and homozygous mice. Tail tissues were collected from these mice for genotyping. All procedures with animals were approved by the University of Manitoba Animal Protocol Management and Review Committee and performed according to guidelines set by the Canadian Council on Animal Care. Polymerase chain reaction (PCR) was used to identify the genotype of transgenic mice.

3.1.1 Genotyping

3.1.1.1 DNA extraction and dilution

Genomic DNA was extracted from tail snips following Wizard Genomic DNA Purification protocol (Promega, A1125). Samples were digested at 55°C overnight in 600 µl of chilled ethylenediaminetetraacetic acid (EDTA)/Nuclei Lysis solution (120 µl of 0.5M EDTA solution (pH 8.0) and 500 µl of Nuclei Lysis Solution) and 17.5 µl of 20 mg/ml proteinase K (Promega, V3021) per sample. After digestion, 200 µl of Protein Precipitation Solution was then added and vortexed. Samples were cooled to room temperature and then centrifuged at 12,000 × g and the protein pellets were discarded. A volume of 600 µl of isopropanol was then

added for DNA precipitation. DNA pellets were formed by centrifugation at 12,000 × g for 20 minutes and were washed using 600 µl of 70% ethanol. The pellets were dried and finally dissolved in 30 µl of DNA Rehydration Solution, which contains 10mM Tris-HCl (pH 7.4) and 1mM EDTA (pH 8.0) by heating to 65°C for one hour.

3.1.1.2 Polymerase chain reaction (PCR)

PCR was performed using DNA extracted from transgenic mice. For hENT1 the forward and reverse primers were 5'-GAG AAC ACC ATC ACC ATG ACA ACC-3' and 5'-GCA GTC CTT CTG TCC ATC CTT TGT-3'. For mouse beta casein, exon 7 (MBCex7), the forward and reverse primers were 5'-GAT GTG CTC CAG GCT AAA GTT-3' and 5'-AGA AAC GGA ATG TTG TGG AGT-3'. The PCR products were expected to be 1.4kb and 0.54kb, respectively. PCR was performed using puReTaq Ready To Go PCR beads (GE Healthcare,27-9557-02), which contains 50 nM deoxynucleoside triphosphates (dNTPs), 1.5mM MgCl2, 2.5 units puReTaq, 0.1-0.2 µM primers and 1 µl of DNA from each sample. PCR consisted of 30 cycles as follows: 95°C for 45 sec, 55°C for 45 sec and 72°C for 3 min. After a final 10 min 72°C elongation step samples were kept at 4°C. Electrophoresis was then performed on 0.8% agarose gel, containing ethidium bromide for visualization, in 1.0X Tris Acetate-EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA) for 30-90 minutes at 105 volt. DNA bands were viewed under ultraviolet light and recorded using a Gel Logic 100 Imaging System (Kodak).

3.1.2 hENT1 radioligand binding assay

3.1.2.1 Membrane preparation

Brain samples were collected after all the experiments were done. Cortex, striatum, cerebellum and hippocampus were dissected from each brain sample and saved in -80°C freezer for later use. For [^{3}H] S-(4-nitrobenzyl)-6-thioinosine ([^{3}H]NBMPR) binding assays, synaptosomes were prepared from cerebral cortex samples. Cortices were first homogenized in approximately 10 volumes of ice-cold sucrose (0.32M) and then centrifuged at 1000 x g for 10 min at 4°C. Pellets were washed twice and all the pooled supernatants were centrifuged (20,000 x g, 1 hr, 4°C). The pellets were resuspended in HEPES buffer composed of 110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES, pH 7.4. Bio-Rad dye binding assays (see below) were performed to determine protein concentrations.

3.1.2.2 Bio-Rad dye binding assay

A volume of 100 mg/ml bovine serum albumin (BSA) was prepared and serial dilutions to 50.00, 25.00, 12.50, 6.25, 3.13, 1.56 and 0.78 µg/ml were prepared, together with a blank of 0 µg/ml. A volume of 160 µl from each concentration of BSA (in duplicate) or the different protein samples (in triplicate) were added into separate wells in a 96 well plate then mixed with 40 µl Bio-Rad dye. After mixture, the plate was put into the synergy HT multi-mode microplate reader (BioTek, USA.) for absorbance measurement. The optical density results from BSA standards were plotted against the concentrations to make a standard curve. Protein concentrations for each brain sample were extrapolated from the standard curve ($r^2 >$ 0.99) and corrected for dilutions.

3.1.2.3 [^{3}H]S-(4-nitrobenzyl)-6-thioinosine ([^{3}H]NBMPR) binding assay

[^{3}H]NBMPR binding assays were determined in an assay volume of 0.9 ml, which contained approximately 30 µg/assay synaptosomal protein, 3 nM [^{3}H]NBMPR, and 0.5 U adenosine deaminase. Radioligands, synaptosomes and the reaction buffer were incubated for 1 hr at room temperature. Non-specific binding was determined in the added presence of 30 µM dipyridamole. Assays were performed in duplicate and terminated by vacuum filtration through GF/B filters and washed twice with ice-cold sodium buffer composed of 118 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 25 mM HEPES, 11 mM glucose and 1mM $CaCl_2$. The filters were then placed in scintillation vials with 5 ml of scintillation cocktail and left in the dark for 12 hours. A scintillation counter was used to determine radioactive counts. Because the protein samples were diluted to approximately 30 µg/assay according to the results of the Bio-Rad dye binding assays, these assays were performed again with the diluted protein samples to determine the final concentration of synaptosomal protein in each assay.

3.1.3 Adenosine receptor radioligand binding

3.1.3.1 Membrane preparation

Synaptosomes were prepared from cerebral cortex and striatum samples that were taken after the end of behavioural experiments. Cortices were homogenized in approximately 10 volumes of ice-cold Tris-HCl buffer (50 mM) that also contained EDTA (1 mM), pH 7.4. Samples were then centrifuged at 20,000 x g for 15 min at 4°C, supernatants were removed

and the pellets were washed with 1 ml of the same buffer and centrifuged again at 20,000 x g for 15 min at 4°C. Pellets were resuspended in 1 ml of Tris-HCl, EDTA buffer. Protein concentrations were determined by Bio-Rad dye binding assays.

3.1.3.2 [^{3}H] 8-Cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) binding assay

Radioligand binding assays were performed in an assay volume of 200 µl containing 50 µl cortex membranes, 50 µl reaction buffer and 100 µl [^{3}H]DPCPX. Radioligand concentrations and membranes were all diluted with Tris-HCl buffer (50 mM; pH 7.4). Reaction buffer was composed of 50 mM Tris-HCl, pH7.4, 2.5 IU adenosine deaminase (ADA) and 0.02% Triton X-100. For non-specific binding, 20 mM theophylline was added (Lorenzo *et al.*, 2010). Assays were incubated for 1 hour at room temperature and then vacuum filtered through GF/B filters and washed twice with ice-cold 50 mM Tris-HCl. Filters were placed in scintillation vials with 5 ml scintillation cocktail and radioactive counts were obtained after at least 12 hr incubation in a dark environment. A volume of 50 µl of each radioligand solution was taken for specific activity counting, in duplicate. Final protein concentrations were determined using the Bio-Rad dye binding assay.

3.1.3.3 [^{3}H]ZM 241385 binding assay

The procedure for [^{3}H]ZM 241385 binding assay was similar to [^{3}H]DPCPX binding assays. [^{3}H]ZM 241385 binding was performed in an assay volume of 200 µl containing 50 µl striatum membranes, 50 µl reaction buffer and 100 µl [^{3}H]ZM 241385 radioligands. Radioligand concentrations and membranes were all diluted with reaction buffer that was composed of 50 mM Tris-HCl, pH7.4, 2.5 IU ADA and 0.02% Triton X-100. For non-specific

binding, 20 mM theophylline (Lorenzo *et al.*, 2010) was added. Assays were incubated for 1 hour at room temperature and then vacuum filtered through GF/B filters and washed twice with ice-cold 50 mM Tris-HCl. Filters were placed in scintillation vials with 5 ml scintillation cocktail and radioactive counts were obtained after at least 12 hr incubation in a dark environment. A volume of 50 µl in duplicate of each radioligand solution was taken for specific activity counting. Final protein concentrations were determined by Bio-Rad dye binding assays.

3.1.4 Behaviour tests

3.1.4.1 Wheel running

A voluntary wheel running experiment was performed to investigate the activity level of the mice. In this experiment, mice, 8-10 weeks of age, were housed individually in a fresh plastic cage equipped with food and water and voluntary access to a wireless running wheel (diameter: 15.5cm. Med Associates Inc, USA.). The wireless running wheel collected the counts of the rotation of the running wheel and sent it to a hub that was connected to the computer. Cages were placed in a quiet and environmentally controlled room with 12/12 h light-dark cycle, in which lights were on between 6:00 am and 6:00 pm and off between 6:00 pm and 6:00 am. In the experiment, mice were placed in the cages at 10:00 am and the counts of wheel rotation were collected for 46 h by the SOF-860 Wheel Manager (Med Associates Inc, USA) system.

3.1.4.2 Rotarod testing

A rotarod treadmill was used to determine ethanol-induced motor incoordination in mice. Mice were trained for over three days before the experiment to run on the rod for 180 s at 20 rpm. Before each experiment, mice were placed in a quiet room for 45 min for acclimatization. During training sessions, mice were first placed on the still rod for 5 min to acclimate to the equipment. Training sessions included two different kinds of trials: acceleration trial and experiment trial. The acceleration trial was a 120 s trial that started at 1.0 rpm and ended at 15 rpm with an acceleration of 1.0 rpm per 2 s. The experiment trial was a 180 s trial with a consistent speed of 20 rpm. In the first day of training session, mice were assigned to run the acceleration trial 3 times and run the experiment trial once. Mice were assigned to run both trials twice in the second day and run the experiment trial 4 times in the third day. Between each trial, mice had 3 min to rest. All the mice were able to stay on the rod for 180 s at 20 rpm after the training. For the caffeine experiments, mice were injected intraperitoneally (i.p.) with 2 g/kg ethanol (20% v/v in saline), or 2 g/kg ethanol plus 6.5 mg/kg caffeine. For the DPCPX experiments, mice were injected (i.p. volume = 5 ml/kg) with DPCPX (1mg/kg) or vehicle that was composed of dimethyl sulfoxide (DMSO), and 15% cremophor EL in saline (El Yacoubi *et al.*, 2000) 5 min prior to the injection of 2g/kg ethanol (20% in saline). Each mouse was placed on the rod immediately after the injection of ethanol and every 15 min for 1 h. The amount of time that each mouse remained on the rod to a maximum of 180 s was recorded.

3.1.4.3 Loss of righting reflex (LORR) experiment for ethanol sensitivity

To test ethanol sensitivity, an experiment was performed to investigate the duration of the loss of righting reflex (LORR) after the intraperitoneal injection of ethanol. In this experiment, mice, 8-10 weeks of age, were placed in an observation chamber in a quiet room for 45 min for acclimatization. After the acclimatization period, mice were injected with ethanol (3.6 g/kg; 20% v/v in saline) and put back into their chamber. Each mouse was gently placed on its back when the locomotion ceased. The time duration of the LORR, which is the duration of time it takes for the mouse to return to its upright posture, was collected for later analysis.

3.1.4.4 Caffeine dose-response relationship

Different doses (0, 6.25, 12.5, 25 and 50 mg/kg) of caffeine were prepared in saline by diluting a stock concentration of 5 mg/ml. Mice were divided into 5 groups by a different person to receive different doses of caffeine in order to maintain a similar group size for both genotypes and to maintain blinding of the investigator to the genotype of the mice. Prior to the experiment, mice were placed in the procedure room for 45 min for habituation. Mice were then injected (0.01 ml/g body weight, i.p.) with different doses of caffeine and their activity was monitored by VersaMax animal activity system (Accu Scan Instrument Inc, USA) for 1 hr. Injection volume was consistent between doses.

3.1.4.5 Caffeine sensitivity

A locomotor experiment was performed to investigate changes in behavioural response after injection of caffeine, i.p. In this experiment, mice, 8-10 weeks of age, were placed

individually in an activity chamber between 9:30 am and 10:30 am. Chambers were equipped with voluntary access to food and water. Each chamber was surrounded by infrared photobeam sensors and the activities of the mice were monitored for a period of 6 h. Mice were left alone in the chambers without treatment in the first 3 h for acclimatization. After the acclimatization period, mice were injected with caffeine (25 mg/kg, i.p. 0.01ml/g) and monitored for a further 3 h. Counts of photobeam interruption were continually collected by a VersaMax Animal Activity System (Accu Scan Instrument Inc, USA) and summed over 20 minute intervals. For the control group, vehicle (saline) was injected (i.p. 0.01ml/g) instead of caffeine. We recorded the number of horizontal photobeam interruptions as horizontal activity. The number of repeated interruptions of a single photobeam was recorded as fine movements, which represents the feeding, grooming, and other repetitive behaviours of mice.

3.1.4.6 N^6-cyclopentyladenosine (CPA) dose-response relationship

Different doses (0, 0.3 and 1mg/kg) of CPA were prepared as solution with DMSO (15%), Cremophor EL (15%) in saline. CPA was first dissolved in DMSO. Cremophor EL was then added and then the remaining volume of saline was added to achieve the desired final concentrations. Similar to the caffeine dose-response experiment, each group of mice received a different dose of CPA. After 45 min of habituation, mice were injected (i.p. 0.01ml/g) with CPA and activities were monitored for 1 hr.

3.2 CD73 Knockout Mice

3.2.1 Behavioural tests

Using methods described above, LORR, caffeine sensitivity, and rotarod experiments were also performed with CD73 knockout and C57BL6 wild type mice.

3.2.2 Anxiety tests

3.2.2.1 Locomotor activity

To test the baseline activity level of the mice, locomotor activity was assessed in a plastic transparent box surrounded by a frame equipped with infrared beams (42 cm×21 cm×20 cm) (AM1052, Benwick Electronics, Essex, UK). Animal activity was recorded automatically by counting the number of beam interruptions. Each mouse was tested for a 10 min session without prior habituation to the apparatus. Data were collected in 2 blocks of 5-min intervals.

3.2.2.2 Open field experiment

The open field box was designed as shown in figure 2, a grey square box (90cm × 90cm) made of compressed wood. The apparatus was partitioned into 36 equal sized squares, with the 20 border squares defined as the outer zone and the 16 central squares called the inner zone. Each mouse was placed in a particular corner of the field and its behaviour was recorded by a video camera hanging from the ceiling for a period of 5 min. Videos were analyzed by

computer using ANY-maze (Stoelting Co, USA) software to get the amount of time that mice spent in the inner zone.

3.2.2.3 Light/dark box experiment

The light/dark box was designed as shown in figure 3. Initially, with the shuttle door closed, the mouse was placed into the dark box and the lid was replaced on top. After 1 min habituation, the shuttle door was lifted open to allow the mouse access to the light box. The amount of time spent in the light box during a 5 min period was measured. The mouse was considered to have entered a box when all four paws were in the box. Behaviours were recorded by a video camera hanging from the ceiling and videos were analysed by computer using ANY-maze (Stoelting Co, USA) software.

3.2.2.4 Elevated T-maze (ETM)

ETM, is a T shaped like wooden apparatus as shown in figure 4. The experiment was recorded using a camcorder that was hooked up above the maze. The behaviour of each mouse was observed from outside the procedure room to reduce additional stress in the environment. Each mouse was first placed on an open arm without access to other arms for 30 min one day prior to the experiment day. On the experiment day, each mouse was first placed in the end of the closed arm; the time taken by the mouse to leave the closed arm with all four paws was recorded three times as the Avoidance 1, 2 and 3 with 30 s inter trial intervals. Following the avoidance testing and a 30 s inter trial interval, the mouse was placed at the end of the open arm and the time that it took to leave the open arm was recorded three times as escape 1, 2 and

3. For each trial there was a cut off time of 300 sec (5 min). Behaviours were recorded by a video camera hanging from the ceiling and videos were analysed by computer using ANY-maze (Stoelting Co, USA) software.

3.2.2.5 Elevated plus maze (EPM)

The maze was designed as shown in figure 5. A camcorder was mounted vertically over the maze, and the behaviour of the mouse was scored from a monitor in an adjacent room. The percentage of time spent in the closed arms was recorded as a measure of anxiety. Each mouse was placed in the central square of the plus-maze facing a closed arm and tested for 5 min duration. The time spent in the open was scored. Behaviours were recorded by a video camera hanging from the ceiling and videos were analysed by computer using ANY-maze (Stoelting Co, USA) software.

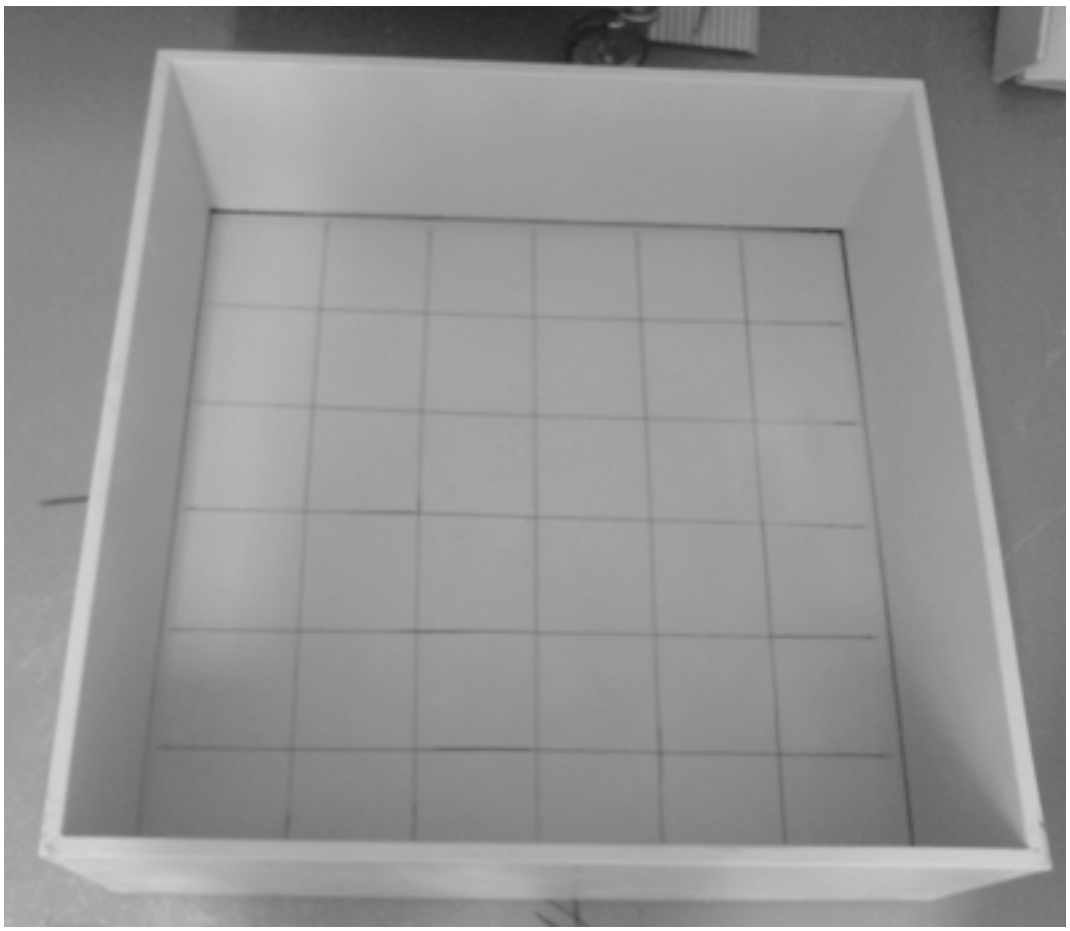


Figure 2. Open Field Experiment Box

The open field test was assessed in a square box (90cm by 90cm) made of compressed wood and painted in grey. The open field box is marked with 36 equal sized squares, of which there were 20 outer border squares and 16 inner zone squares

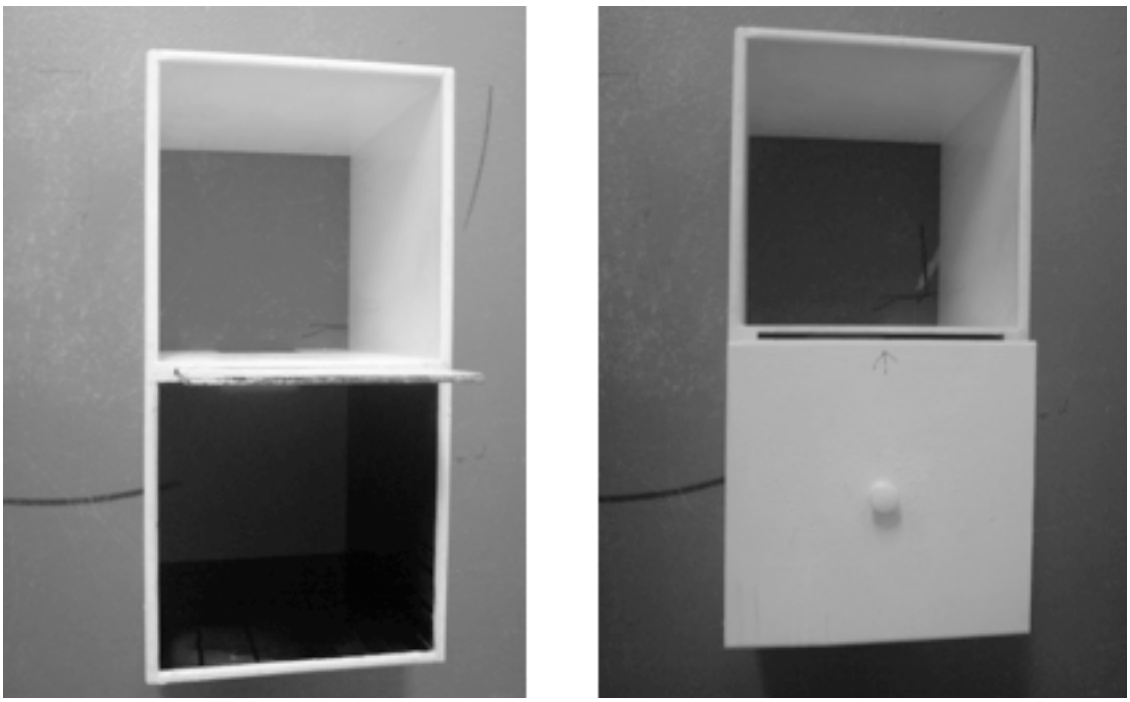


Figure 3. Light/dark Experiment Box

The light/dark box test consisted of two equal sized wooden chambers (25x25cm; and 30 cm height) connected by a shuttle door (10x10 cm) located in the center of the partition at floor level. The light box was open at the top, painted white, and illuminated with a 60 W (400 lux) room light source above the apparatus. The dark box was painted black and had a removable black lid. The shuttle door was raised and lowered manually.

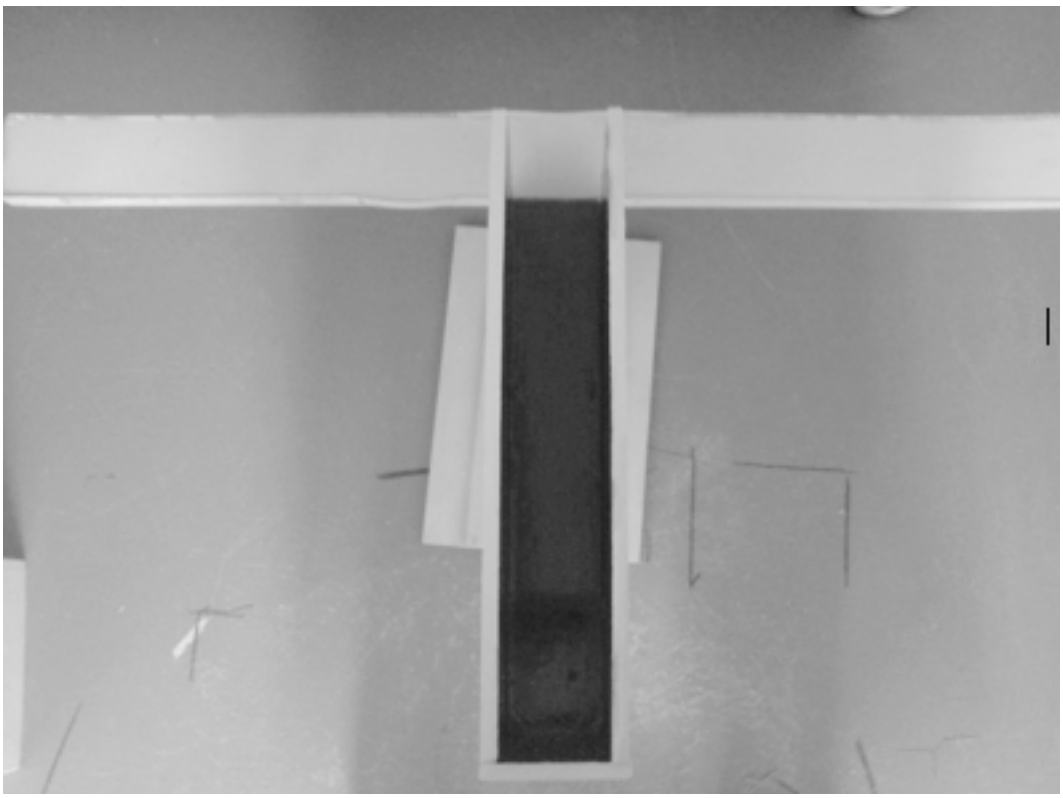


Figure 4. Elevated T Maze

The ETM, was a T shaped wooden apparatus composed of 3 arms with equal dimensions (50 × 12 cm) elevated at 50 cm above the ground. One of the arms was surrounded by three 20 cm high walls.

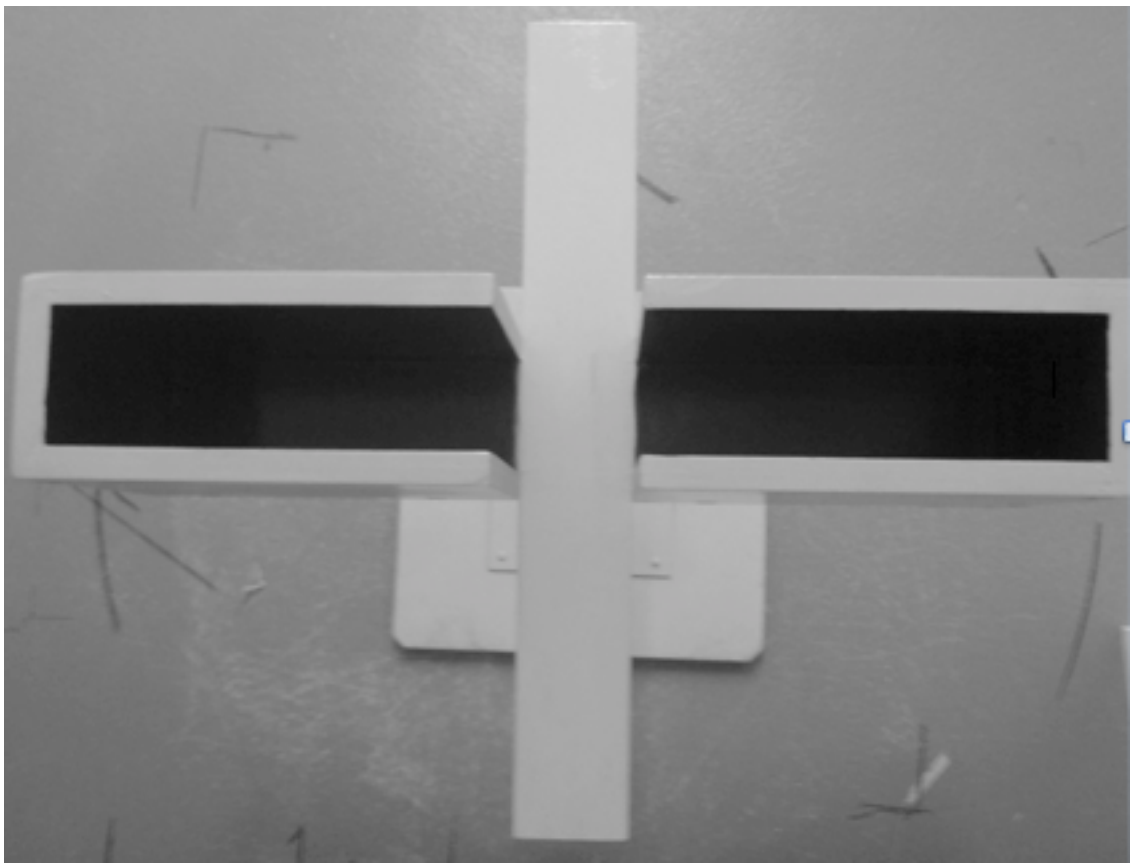


Figure 5. Elevated Plus Maze

The EPM consisted of two open arms (50 ×10 cm), and two closed arms (enclosed by 40 cm high walls) that were connected by a central platform (10 ×10 cm). The maze was elevated at 50 cm from the floor and lit by dim room light.

Chapter 4. RESULTS

4.1 hENT1 transgenic mice

4.1.1 Genotyping

4.1.1.1 PCR

PCR was performed routinely for the identification of wild type and heterozygous transgenic mice, using genomic DNA extracted from mouse tail snips. The expression of hENT1 was positive in heterozygous transgenic mice, but negative for wild type mice. The predicted product sizes from sequence specific primers to mouse beta casein and hENT1 are 0.54 and 1.4kb, respectively. Figure 6 is an example of the result from agarose gel electrophoresis of PCR products. From this gel, lane 2, 3, and 4 can be identified as wild type while lane 1 can be verified as transgenic mouse.

4.1.2 Radioligand binding assays

4.1.2.1 [^{3}H]NBMPR binding assays

[^{3}H]NBMPR, a high affinity and selective radioligand for ENT1, was used to determine the expression of hENT1 in wild type (n=2), heterozygous (n=2), and homozygous (n=2) transgenic mice brain cortex (Figure 7). The isolated mice synaptosomes were put in 3 nM of [^{3}H]NBMPR and reaction buffer for incubation for 1 hr. The results were analyzed for the different genotypes. The binding site density was significantly increased from wild type to transgenic mice. The average increase in the binding site density in heterozygous transgenic mice relative to their wild type littermates was approximately 4 fold, whereas the increase of

homozygous relative to wild type mice was approximately 7 fold. The increase from homozygous relative to heterozygous transgenic mice was also significant.

4.1.2.2 [^{3}H]DPCPX binding assays

[^{3}H]DPCPX, a selective radioligand for the adenosine A_1 receptor, was used for the determination of the A_1R density in the wild type (n=4), and heterozygous (n=4) transgenic mice cortical tissue. The isolated synaptosomes from mice cortex were incubated with eight different concentrations (0.006 - 4 nM) of [^{3}H]DPCPX. Data were pooled and plotted as saturation curves (Figure 8). From the results the total number of the receptors (Bmax) were 1284 and 1762 fmol/mg protein in wild type and heterozygous transgenic mice, respectively. The respective average equilibrium dissociation constants (Kd) were 0.56 and 0.68 nM. The saturation curves showed a trend towards an increase of A_1R in transgenic mice cortex, but this was not statistically significant.

4.1.2.3 [^{3}H]ZM 241385 binding assays

In order to determine the level of adenosine A_{2A} receptors in mice striatum, [^{3}H]ZM 241385, a selective radioligand for A_{2A}R was used for binding assays. The isolated synaptosomes from wild type (n=4) and heterozygous (n=4) mice striatum were incubated in six different concentration (0.05 – 1.5 nM) of [^{3}H]ZM 241385. Data were pooled and plotted into saturation curves (Figure 9). From the results the total number of the receptors (Bmax) were 5184 and 6372 fmol/mg protein in wild type and heterozygous transgenic mice, respectively. The respective average equilibrium dissociation constants (Kd) were 0.28 and 0.40 nM.

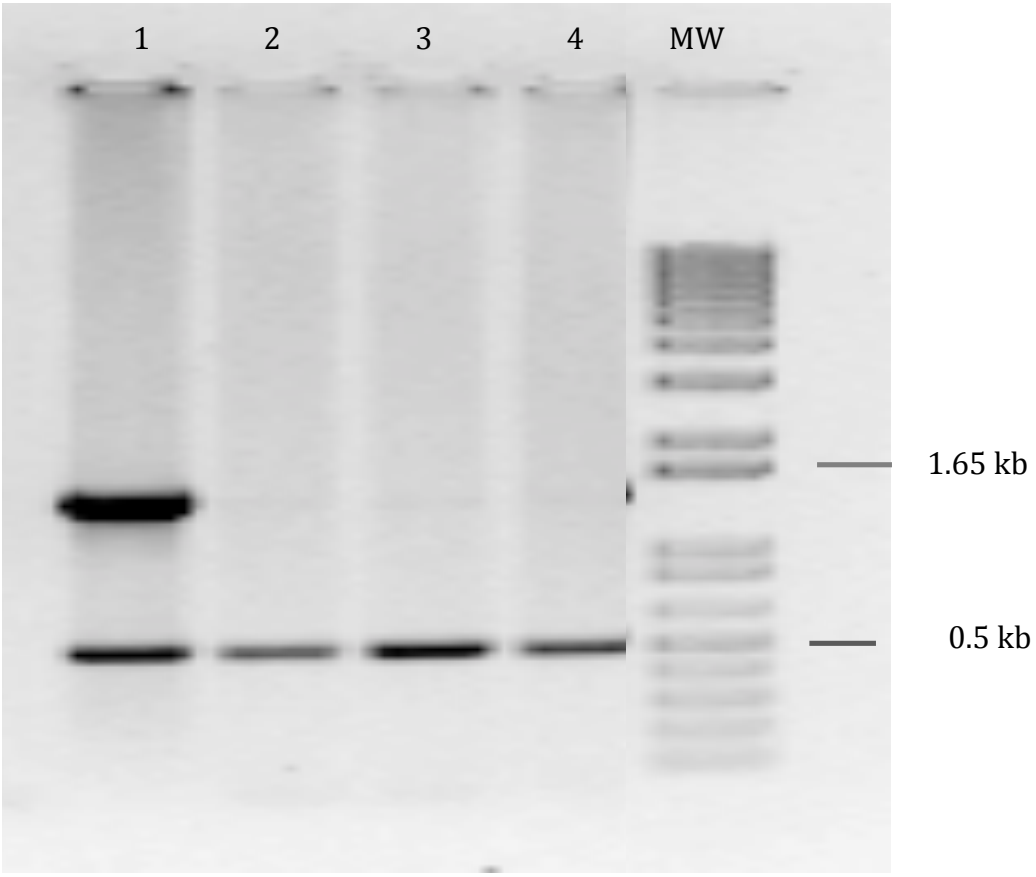


Figure 6. Identification of Transgenic Mice

For the identification of transgenic mice, PCR was performed routinely. In this experiment, genomic DNA was extracted from mice tail snips. Multiplex PCR was performed with sequence specific primers to mouse beta casein (a control gene) and hENT1 and the predicted product sizes were 0.54 and 1.4kb, respectively. From this experiment, lane 1 is transgenic mouse and lane 2, 3, and 4 are all wild type mice.

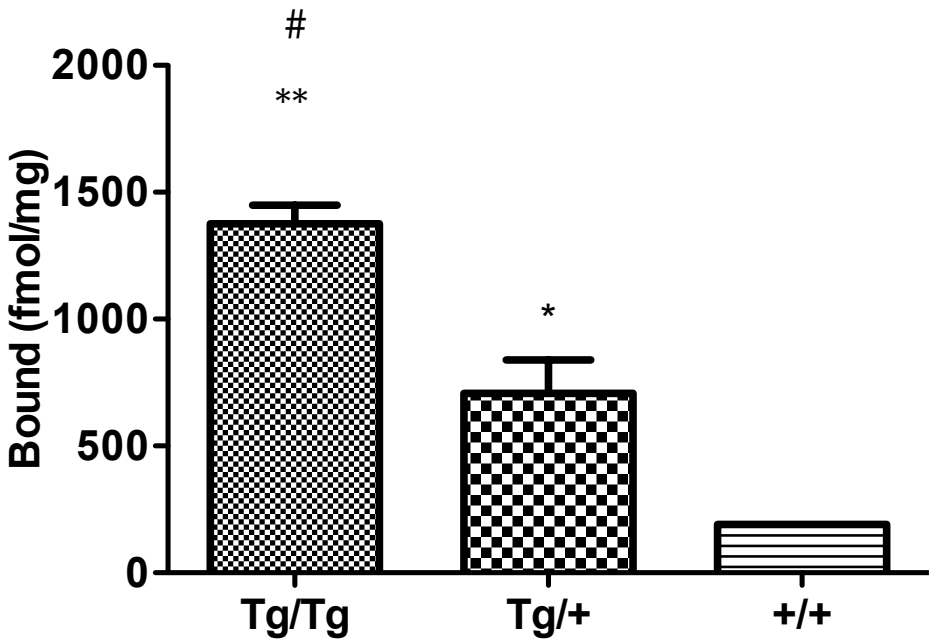


Figure 7. hENT1 [^{3}H]NBMPR Binding Data

[^{3}H]NBMPR binding assays were used to determine the difference in expression of hENT1 protein in mice cortex. In this experiment, synaptosomes were isolated from mouse cortex tissues and incubated with 3 nM [^{3}H]NBMPR in duplicate at 22°C. The average increase in binding site density in heterozygous transgenic mice (Tg/+, n=2) relative to their wild type (+/+, n=2) littermates was approximately 4 fold while the increase of homozygous (Tg/Tg, n=2) was approximately 7 fold. The differences between genotypes were statistically significant. Data are means ± SEM $^{*}p<0.05$, $^{**}p<0.01$, relative to wild type, $^{\#}p<0.05$, relative to heterozygous. ANOVA, Tukey's post tests.

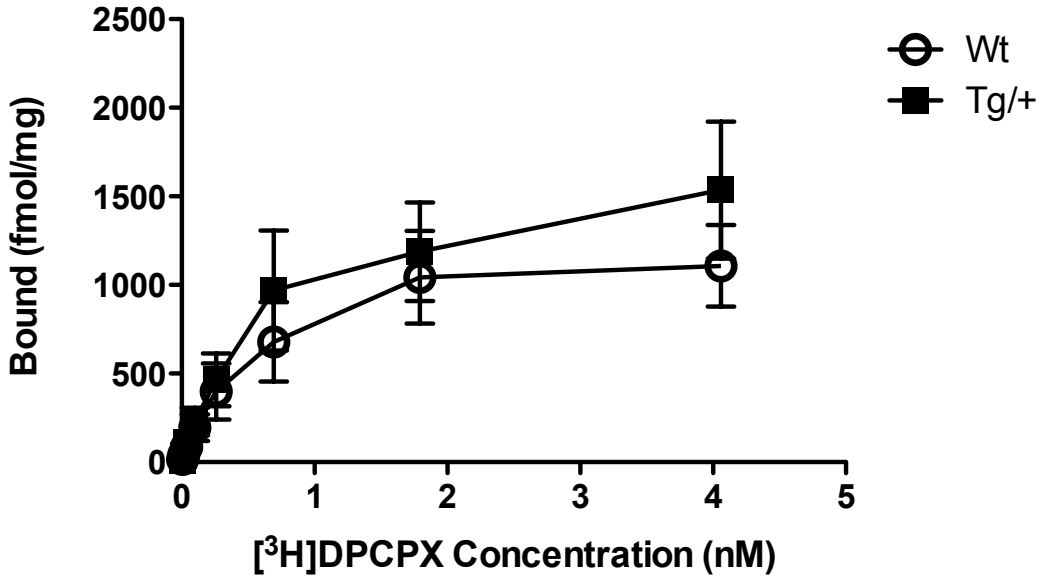


Figure 8. [^{3}H]DPCPX Binding Data In hENT1 Transgenic Mice

[^{3}H]DPCPX binding assays were used to test for a difference in levels of A_1R between wild type (Wt, n=4 mice) and heterozygous (Tg/+, n=4 mice) transgenic mice cortex. Synaptosomes isolated from mice cortex were incubated with different concentrations of [^{3}H]DPCPX at 22˚C. The data showed a trend towards an increase in binding site density of A_1R in heterozygous transgenic mice relative to wild type littermates. No statistically significant differences were detected. Data are means ± SEM.

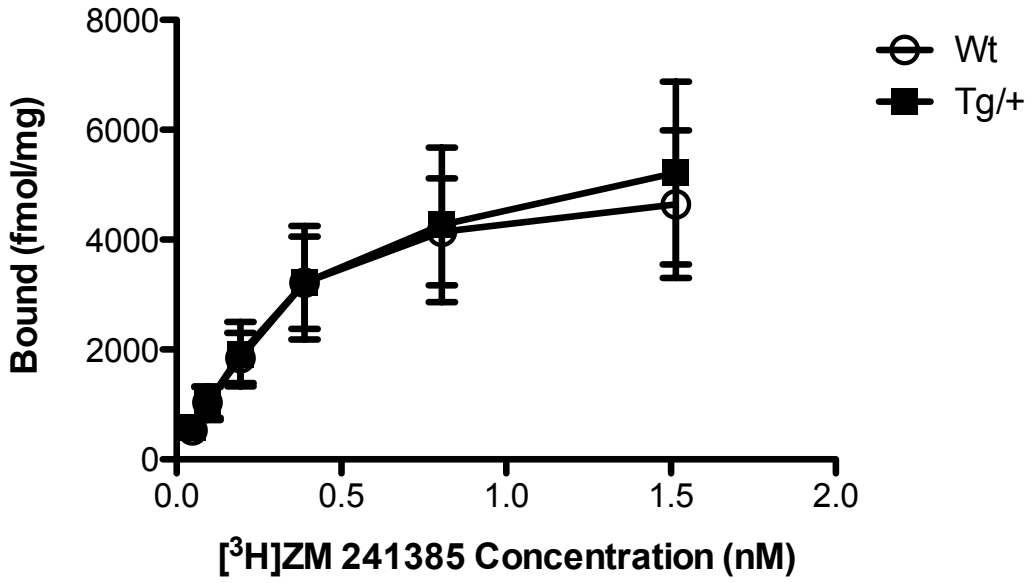


Figure 9. [^{3}H]ZM 241385 Binding Data In hENT1 Transgenic Mice

[^{3}H]ZM 241385 binding assays were used to determine the difference in abundance of $A_{2A}R$ in striatum of wild type (Wt, n=4 mice) and heterozygous (Tg/+, n=4 mice) transgenic mice. Synaptosomes isolated from mice striatum were incubated with different concentrations of [^{3}H] ZM241385 at 22˚C. The results showed similar density of $A_{2A}R$ in hENT1 and wild type mouse striatum and no significant difference was observed. Data are means ± SEM.

4.1.3 Behaviour experiments

A previous study reported that the behavioural responses to caffeine and ethanol were altered between wild type littermates and heterozygous hENT1 transgenic mice (Parkinson *et al.*, 2009). In the present study, the hypothesis that homozygous transgenic mice had increased behavioural response to heterozygous littermates was tested.

4.1.3.1 Wheel running experiment

In wheel running experiments, mice were placed in an environmentally controlled room with voluntary access to a wireless running wheel for 46 h. All mice used the wheel extensively during the night but not the day time (Figure 10A). For wild type mice, but not transgenic mice (Figure 10B), wheel running increased significantly ($p<0.05$) from the first night to the second night of access. No significant difference was detected between heterozygous and homozygous mice.

4.1.3.2 Rotarod testing

Ethanol has an effect to produce motor incoordination, and rotarod is a test that determines motor incoordination. Wild type, heterozygous, and homozygous transgenic mice were injected (i.p.) with 2 g/kg ethanol (Figure 11A) or 2 g/kg ethanol plus 6.5 mg/kg caffeine (Figure 11B). Immediately and every 15 min for 1 h, mice were put on the rod; latency of falls was recorded and plotted. No difference was found for the effect of caffeine between genotypes.

To test the effect of an A_1R selective antagonist, mice were injected (i.p.) with vehicle or 1mg/kg DPCPX 5 min prior to the injection of ethanol. Mice were put on the rod immediately and every 15 min for 1 h. Latency of falls were recorded. DPCPX increased the latency of falls in both genotypes compared to vehicle, but no difference was found between genotypes (Figure 11C).

4.1.3.3 LORR experiment for ethanol sensitivity

Ethanol injections (3.6 g/kg, i.p.) had a hypnotic effect on mice (Figure 12) and induced LORR in mice. In this experiment, LORR duration was significantly increased in both homozygous and heterozygous mice relative to wild type mice ($p<0.05$). A trend towards an increased effect of ethanol in homozygous relative to heterozygous mice was observed but did not reach statistical significance.

4.1.3.4 Caffeine sensitivity

Caffeine injections (25 mg/kg, i.p.) increased locomotor activity in both wild type and transgenic mice, with a significantly greater stimulation of horizontal activity (Figure 13C) and fine movements (Figure 13D) in wild type mice than transgenic mice. No significant differences were detected between heterozygous and homozygous mice. Saline was injected (i.p.) as control and no difference was found between wild type mice and heterozygous transgenic mice (Figure 13A, B).

4.1.3.5 Caffeine dose response relationship

Mice were injected (i.p.) with different doses of caffeine. Immediately after injection, horizontal activity of mice was collected and data were analysed as shown in Figure 14. Lower doses of caffeine (6.25 and 12.5 mg/kg) showed a trend toward increased locomotor activity in both wild type mice and the heterozygous transgenic mice, while injection of caffeine in high dose (50 mg/kg) decreased activity significantly in both groups.

4.1.3.6 CPA dose response relationship

Mice were injected (i.p.) with different doses of CPA (0, 0.3 and 1mg/kg). Counts of horizontal activity of mice were collected and data were analysed as shown in Figure 15. After the injection of CPA (0.3 and 1 mg/kg), a significant decrease of activities occurred in both wild type and heterozygous transgenic mice. No statistically significant differences between genotypes were noted.

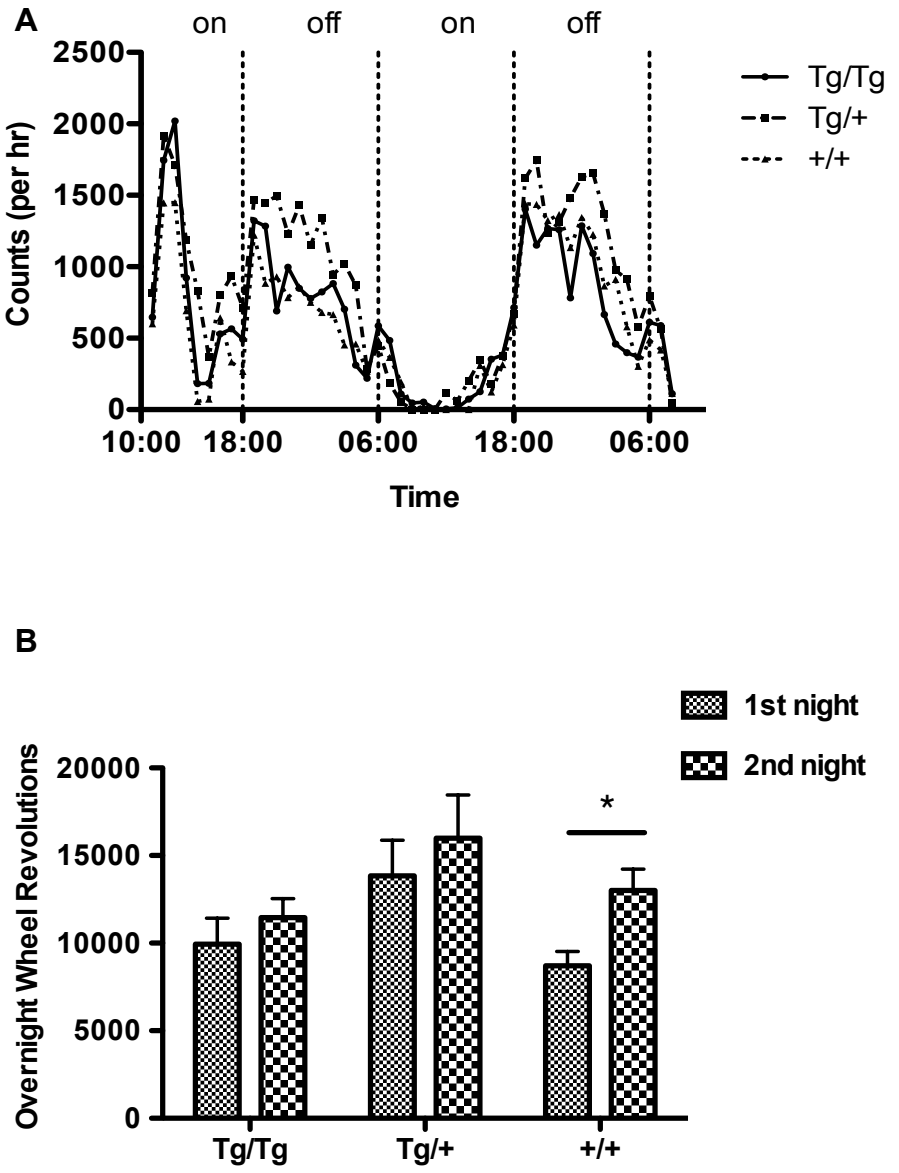
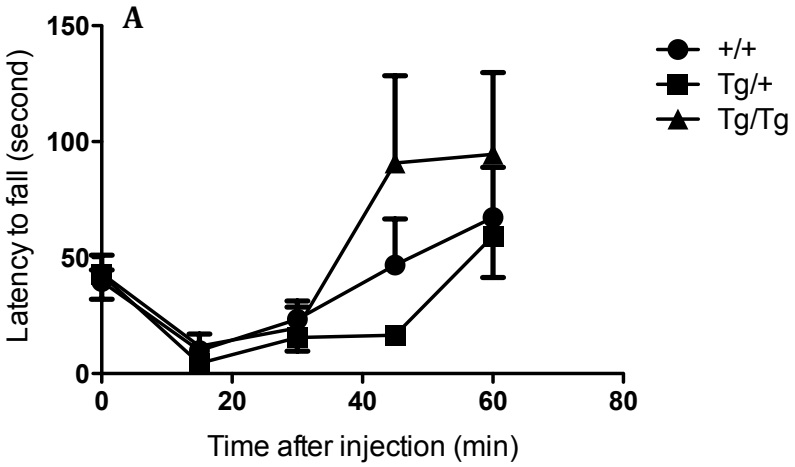
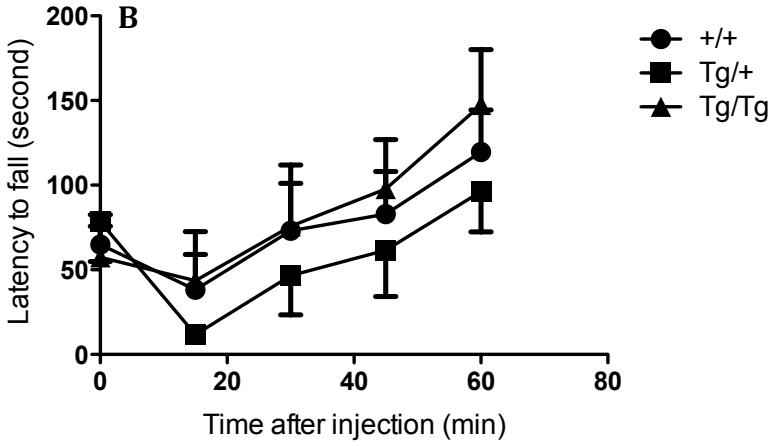


Figure 10. hENT1 Wheel Running Behaviour Data

Wild type (+/+, n=7), heterozygous (Tg/+, n=8) and homozygous (Tg/Tg, n=10) transgenic mice were given voluntary access to a running wheel. A. Hourly wheel revolution counts were collected for 46 hr, light (on) and dark (off) intervals are indicated. B. Overnight wheel revolutions were monitored over two consecutive nights. The total revolutions of each night (18:00 to 06:00) are plotted. Data are means ± SEM, $^{*}p<0.05$, two tailed, t-test.


A
Latency to fall (second)
150
100
50
0
0
20
40
60
80
Time after injection (min)
+/+
Tg/+
Tg/Tg


B
Latency to fall (second)
200
150
100
50
0
0
20
40
60
80
Time after injection (min)
+/+
Tg/+
Tg/Tg

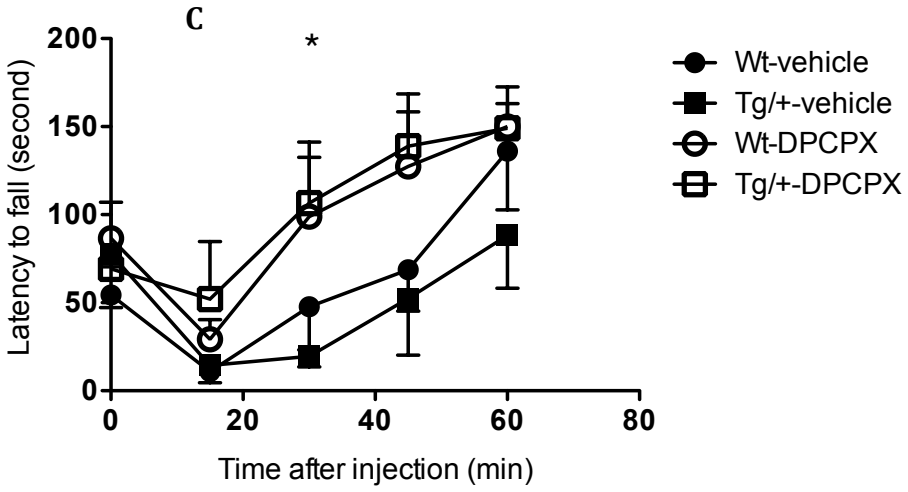

C
*
Latency to fall (second)
200
150
100
50
0
0
20
40
60
80
Time after injection (min)
Wt-vehicle
Tg/+-vehicle
Wt-DPCPX
Tg/+-DPCPX

Figure 11. Rotarod Tests

Wild type (+/+, n=8), heterozygous (Tg/+, n=6), and homozygous (Tg/Tg, n=5) transgenic mice were injected with 2 g/kg ethanol (A) or 2 g/kg ethanol + 6.5 mg/kg caffeine (B) and immediately put on the rotarod, the latency time to fall was recorded. Caffeine reduced the ataxia effects of ethanol. No statistically significant difference was found in the latency between genotypes after the above injections. Data are mean ± SEM (ANOVA, Tukey's post tests). In the DPCPX experiment (C), mice were injected with DPCPX 5 min prior to the injection of ethanol. DPCPX produced a trend toward a reduced motor incoordination effect produced by ethanol, with statistically significant differences found at 30 min after injection in transgenic mice. Data are means ± SEM, $^{*}p<0.05$, transgenic mice with DPCPX injections relative to transgenic mice with vehicle injections, two tailed, t-test.

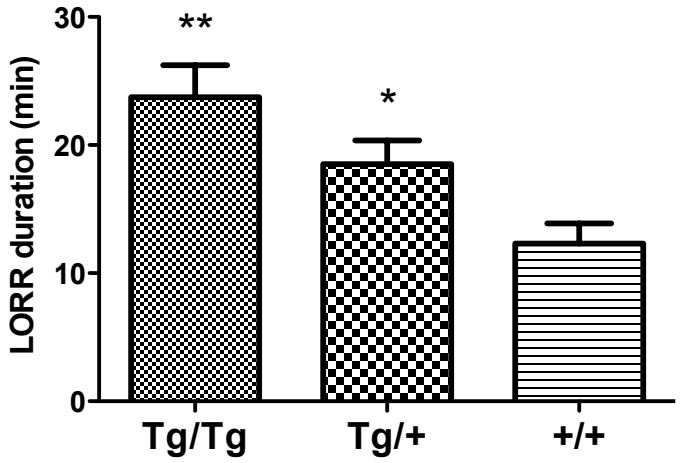


Figure 12. hENT1 Ethanol Sensitivity Data

Wild type (+/+, n=16), heterozygous (Tg/+, n=34), and homozygous (Tg/Tg, n=27) transgenic mice were injected with 3.6 g/kg ethanol (i.p.) and LORR duration was determined. Data are means ± SEM $^{*}p<0.05$, $^{**}p<0.01$, relative to wild type, ANOVA, Tukey's post tests.

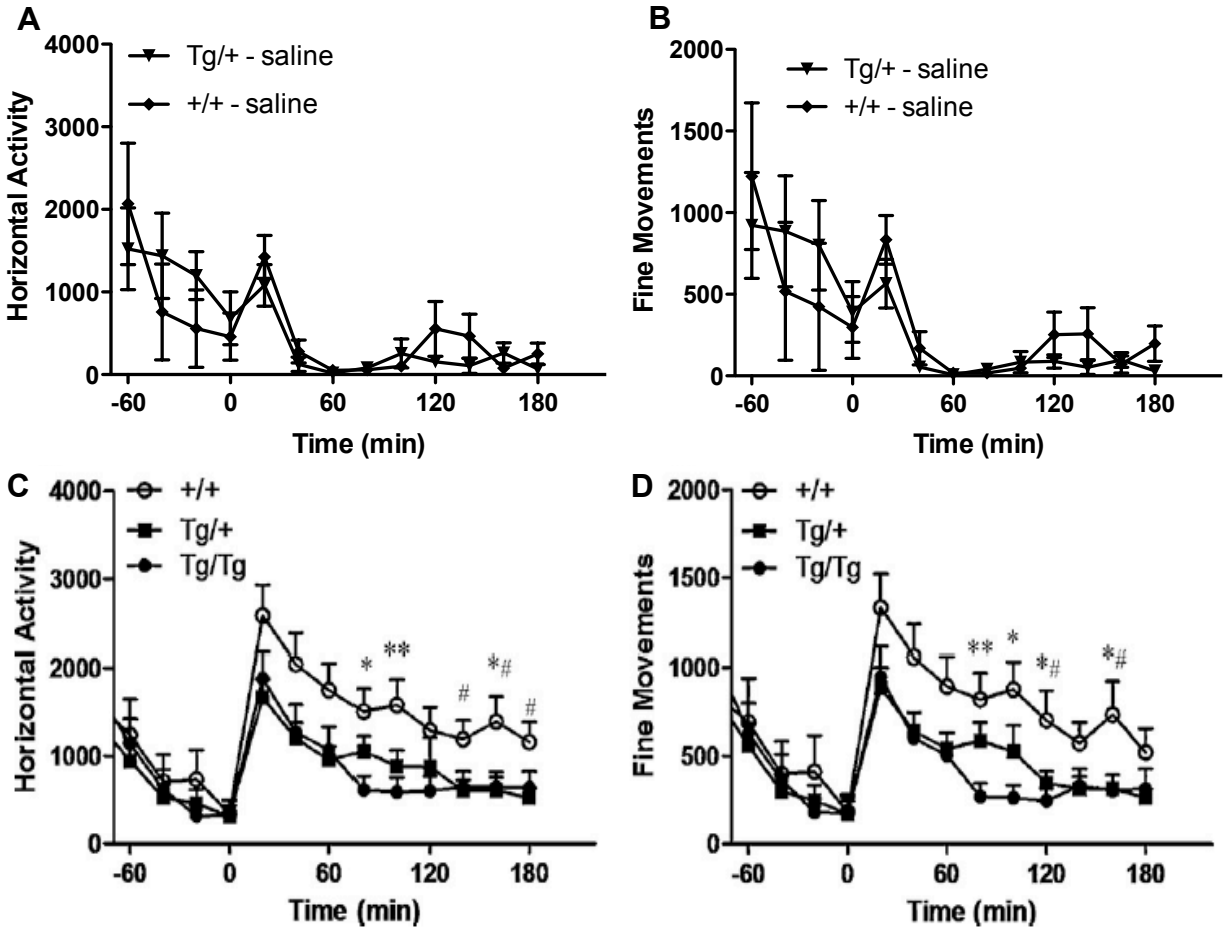


Figure 13. hENT1 Caffeine Sensitivity Open Field Behavioural Experiment

Homozygous or heterozygous hENT1 transgenic or wild type mice were placed in activity chambers. After an acclimation period at time=0 min, wild type (+/+, n=6), and heterzygous (Tg/+, n=8) transgenic mice were injected (i.p.) with saline (A, B) as control. Wild type (+/+, n=14), heterozygous (Tg/+, n=29), and homozygous (Tg/Tg, n=21) transgenic mice were injected (25 mg/kg, i.p.) with caffeine (C, D) at t=0 min. Horizontal activity (A, C) is detected by interruptions in adjacent infrared photobeams. Fine movements (B, D) are detected by repeated interruptions of the same photobeam. Data are means ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, relative to Tg/Tg; $^{\#}p<0.05$, relative to Tg/+ (ANOVA, Tukey's post-tests).

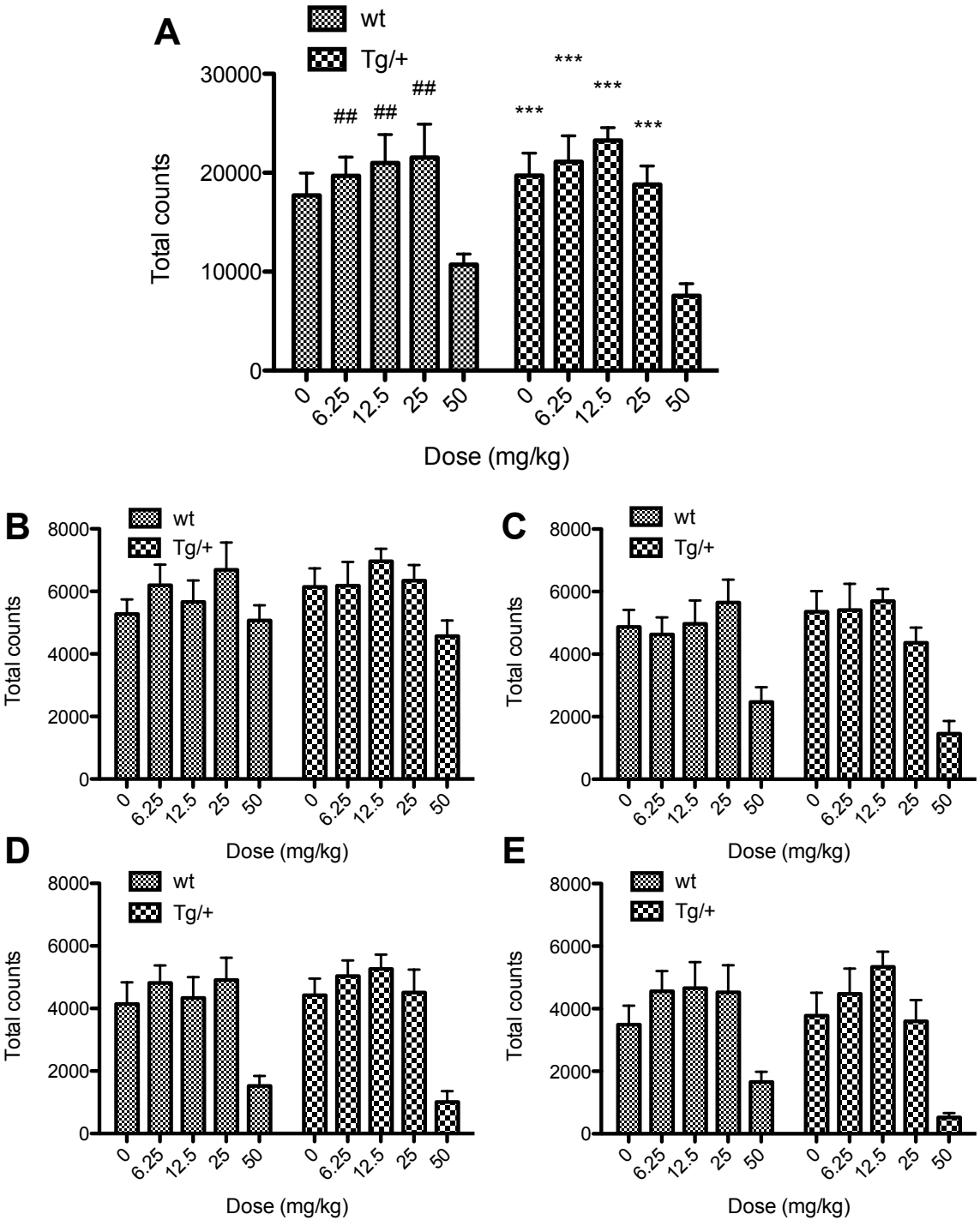

A
wt
Tg/+
Total counts
30000
20000
10000
0
##
##
##
***
***
***
0
6.25
12.5
25
50
Dose (mg/kg)
B
C
D
E

Figure 14. Caffeine Dose Response Relationship

Wild type (wt) mice were injected (i.p.) with 0 (n=8), 6.25 (n=11), 12.5 (n=9), 25 (n=7) or 50 mg/kg (n=8) of caffeine in saline. Heterozygous transgenic (Tg/+) mice were injected with 0 (n=8), 6.25 (n=8), 12.5 (n=9), 25 (n=8) or 50 mg/kg (n=9) of caffeine in saline. Locomotor activity of mice was monitored during 15 min intervals for 1 h after injections. Horizontal activity is shown for 0-60 min (A), 0-15 min (B), 15-30 min (C), 30-45 min (D) and 45-60 min (E). Significantly reduced activity was obtained with 50mg/kg caffeine in both wild type and transgenic mice. No statistically differences between genotypes were detected. Data are means ± SEM, $^{***}p<0.001$, relative to 50 mg/kg in Tg/+ mice; $^{\#\#}p<0.01$, relative to 50 mg/kg wt (Two way ANOVA, Bonferroni post-tests).

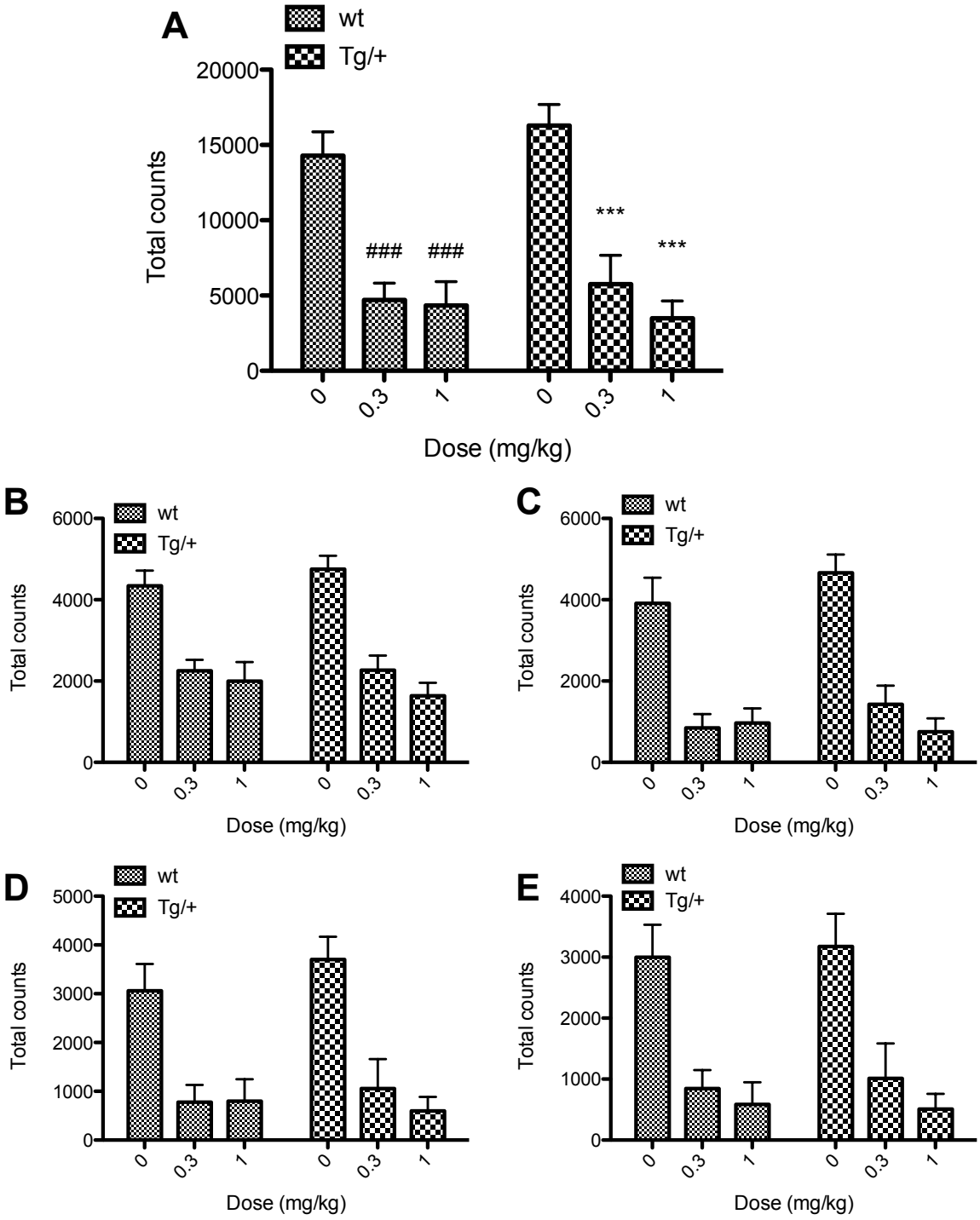

A
wt
Tg/+
Total counts
20000
15000
10000
5000
0
###
###
***
***
0
0.3
1
0
0.3
1
Dose (mg/kg)
B
wt
Tg/+
Total counts
6000
4000
2000
0
0
0.3
1
0
0.3
1
Dose (mg/kg)
C
wt
Tg/+
Total counts
6000
4000
2000
0
0
0.3
1
0
0.3
1
Dose (mg/kg)
D
wt
Tg/+
Total counts
5000
4000
3000
2000
1000
0
0
0.3
1
0
0.3
1
Dose (mg/kg)
E
wt
Tg/+
Total counts
4000
3000
2000
1000
0
0
0.3
1
0
0.3
1
Dose (mg/kg)

Figure 15. CPA Dose Response Relationship

Wild type (wt) mice were injected (i.p.) with 0 (n=14), 0.3 (n=16) or 1 mg/kg (n=14) of CPA. Heterozygous transgenic (Tg/+) mice were injected with 0 (n=12), 0.3 (n=13) or 1 mg/kg (n=13) of CPA. Locomotor activity of mice was monitored for 1 h after injections. The counts of horizontal activity are shown for 0-60 min (A), 0-15 min (B), 15-30 min (C), 30-45 min (D) and 45-60 min (E). Significantly reduced activity was obtained with 0.3 and 1 mg/kg CPA in both wild type and transgenic mice. No statistically significant differences between genotypes were noted. Data are means ± SEM, $^{***}p<0.001$, relative to 0 mg/kg in Tg/+ mice; $^{###}p<0.001$, relative to wt (Two way ANOVA, Bonferroni post-tests).

4.2 CD73 knockout mice

4.2.1 Behavioural tests

LORR, caffeine sensitivity, and rotarod experiments were performed on CD73KO mice and C57Bl6 wild type mice.

In the LORR experiments, CD73KO and wild type mice were injected (3.6 g/kg, i.p.) with ethanol and their LORR duration was recorded. Ethanol produced a significantly greater hypnotic effect in CD73KO mice ($p<0.001$) compared to wild type mice (Figure 16).

In the caffeine sensitivity experiments, CD73KO and wild type mice were injected (25 mg/kg, i.p.) with caffeine at t=0 min, and their horizontal activity and fine movements were monitored. CD73KO mice showed significantly higher levels of horizontal activity and fine movements before injections, compare to wild type mice. No differences between CD73KO and wild type mice were noted after caffeine injections (Figure 17).

In the rotarod experiments, CD73KO mice and wild type mice were injected with different doses (1 g/kg, 1.5 g/kg, 2 g/kg) of ethanol and immediately put on the rotarod. The latency time to fall off the rod was recorded. Injection (i.p.) of 1 g/kg ethanol produced a significantly longer latency of fall in CD73KO compared to wild type mice at 30 min. Longer latency was found in CD73KO relative to wild type mice 60 min after injection (i.p.) of 1.5g/kg ethanol. No differences were obtained at the dose of 2g/kg (Figure 18).

4.2.2 Anxiety tests

To test anxiety-like behaviours of the mice, we performed a set of experiments including locomotor activity, open field test, light/dark box test, elevated plus maze and elevated T maze test. We examined the baseline locomotor activity level for a 10 min duration using 18 male mice of each genotype. These results indicate that CD73KO mice show normal baseline activity level compared with the wild type mice (Figure 19). For the open field test, highly illuminated, novel, open spaces are all anxiogenic stimuli for mice. The mice have a natural aversion (Crawley *et al.*, 1980) to be in the center of the open field box, so we compared the activity in the inner zone vs. the outer zone as an indication of anxiety-like behaviour. Our data show that CD73KO mice spent a significantly greater amount of time in the inner zone compared with wild type mice (Figure 20, P=0.0027, two tailed, t test).

The light/dark box test is another common method to assess anxiety-like behaviours in mice. Bright lights and white surfaces are potentially more anxiogenic than a dark enclosure. Our data show a trend for CD73 KO mice to stay longer in the light box than wild type mice, but this was not statistically significant (Figure 21, P=0.1508, two tailed, t test).

We next performed an ETM test, which investigates the anxiety-like behaviour of the mice. The ETM introduces height and open space as anxiogenic stimuli. Avoidance and escape were both tested three times. In both tests, shortest latencies were observed in the first trial, longer latency in the second trial, and longest in the last. In comparisons between genotypes, a significant reduction of avoidance time was found in the CD73KO mice (Figure 22A), whereas no significant difference was found in the escape time (Figure 22B), relative to wild type mice.

Finally, we examined anxiety-like behaviour using an EPM. EPM exploits the conflict between the tendency to explore a novel area and the aversions to open areas and heights (Pellow *et al.*, 1985). The amount of time each mouse spent in the open arm was collected. CD73KO mice spent significantly greater amount of time in the open arm compared with wild type mice (Figure 23, P=0.0013).

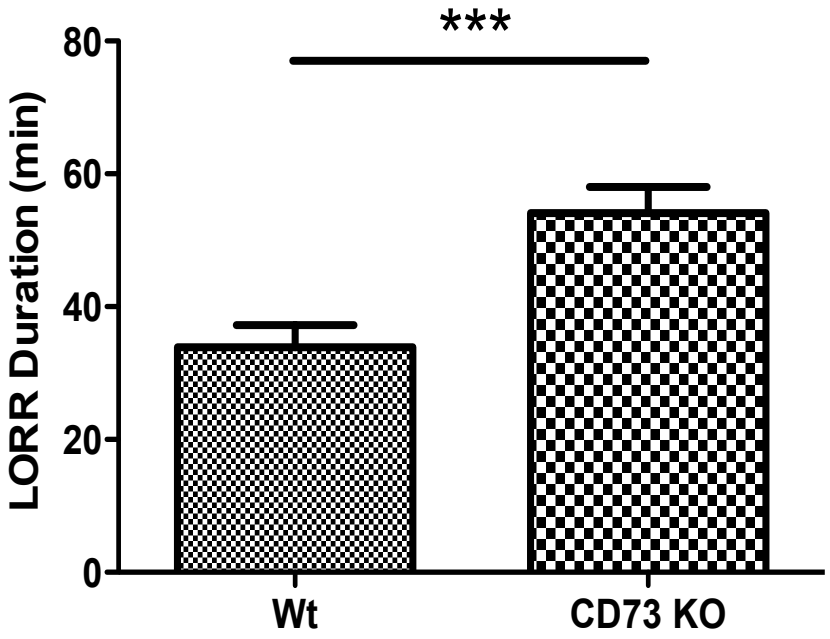


Figure 16. LORR Duration Of CD73KO Mice

CD73 KO (n=16) and wild type (n=14) mice were injected with 3.6 g/kg ethanol (i.p.). When locomotion ceased, mice were gently placed on their backs and the duration of LORR was recorded. CD73 knockout mice showed a significantly longer duration of LORR relative to wild type mice. Data are means ± SEM, $^{***}p<0.001$, two tailed, t test.

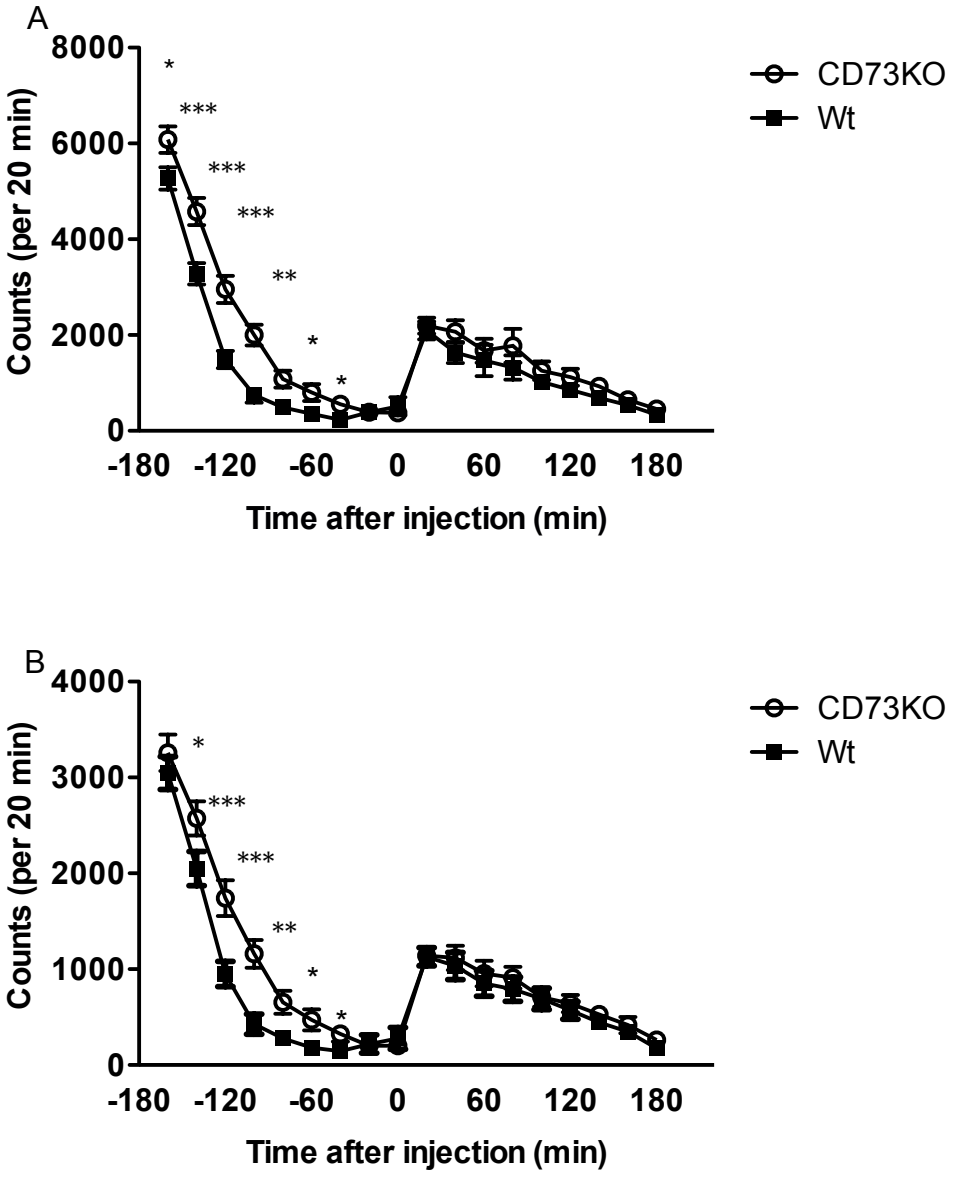


Figure 17. Caffeine Sensitivity Of CD73 KO Mice

CD73 KO (n=28) and wild type (n=32) mice were injected (25 mg/kg, i.p.) with caffeine at t=0 min. Horizontal activity (A) and fine movements (B) were detected. Data are means ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, relative to wild type mice, two tailed, t test.

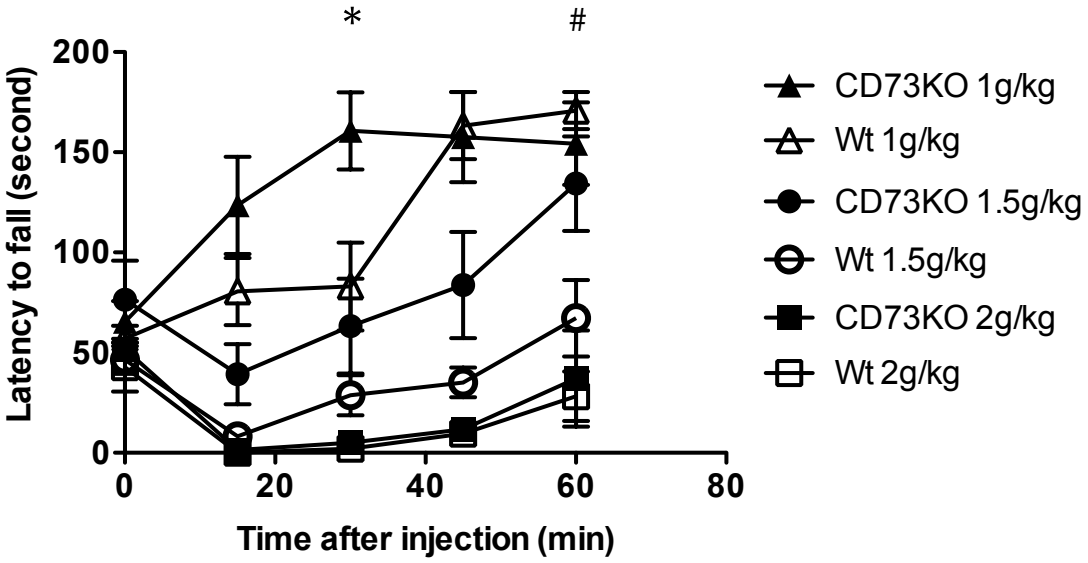


Figure 18. Rotarod Tests CD73KO Mice

CD73KO mice (n=7) and wild type (n=7) mice were injected with different doses (1 g/kg, 1.5 g/kg, 2 g/kg) of ethanol, immediately (time=0) put on the rotarod, and the latency time to fall was recorded. Significant differences were found in the latency to fall after injection of ethanol (1 g/kg, 1.5 g/kg). No difference was noted after injection of 2 g/kg ethanol. Data are means ± SEM, $^{*}p<0.05$, CD73KO (1g/kg ethanol) relative to wild type mice (1 g/kg ethanol), $^{\#}p<0.05$, CD73KO (1.5 g/kg ethanol) relative to wild type mice (1.5 g/kg ethanol), two tailed, t test.

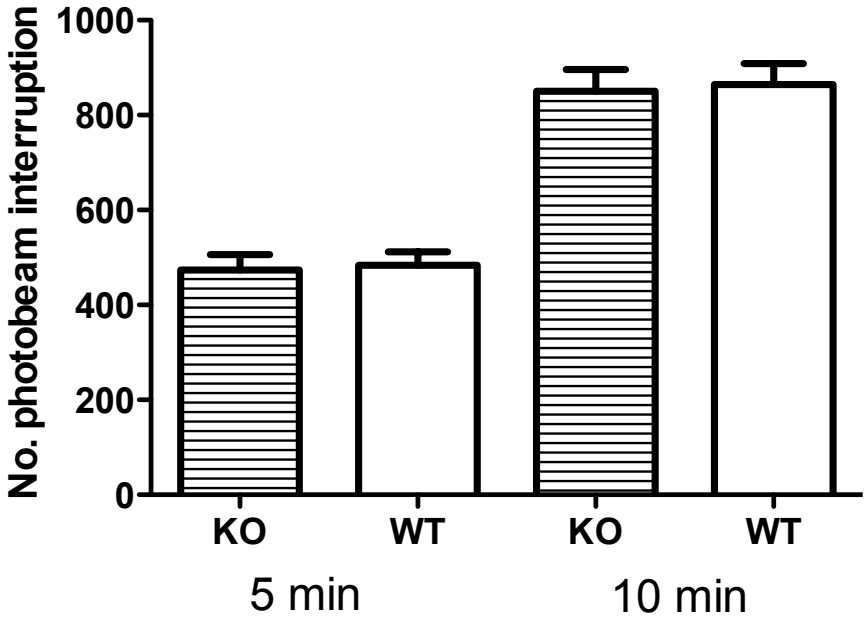


Figure 19. CD73KO Locomotor Data

Baseline locomotor activity was determined by placing CD73KO (KO, n=18) and wild type (WT, n=18) mice in an activity chamber equipped with infrared beams for 10 minutes. The number of infrared photobeam interruptions was recorded after 5 min and again after 10 min. No significant differences were found between genotypes. Data are means ± SEM.

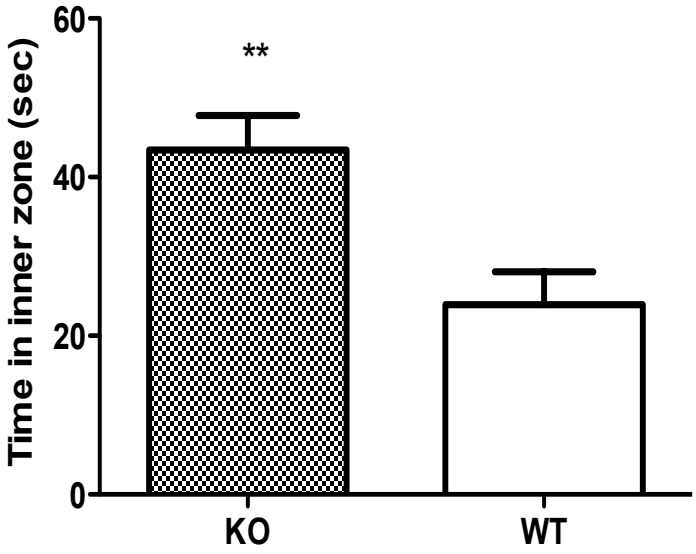


Figure 20. CD73KO Open Field Data

The CD73KO mice show reduced anxiety-like behaviour in the open field. CD73KO and wild type mice were placed in the open field box (36" by 36") and their activities were monitored for 5 min. The time spent in the inner 12 (12" by 12") squares was recorded. The CD73KO mice (KO, n=18) spent a significantly greater amount of time in the inner zone compared with wild type mice (WT, n=18). Data are means ± SEM, $^{**}p<0.01$, two tailed, unpaired t test.

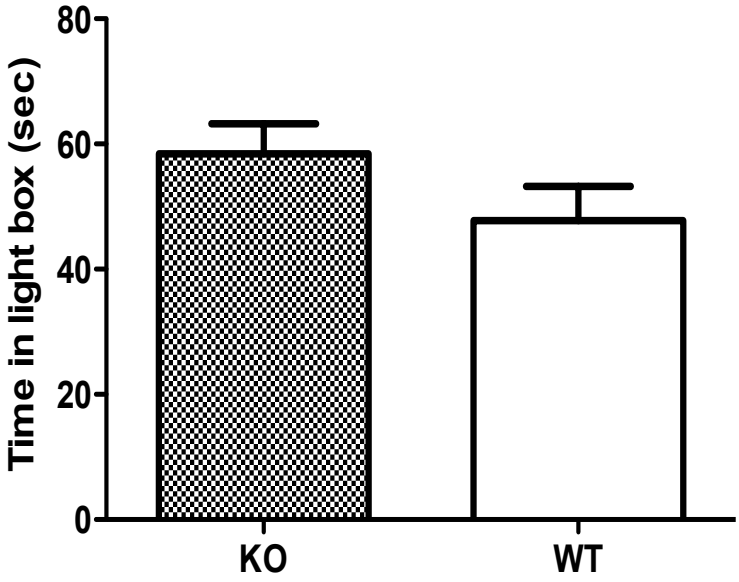


Figure 21. CD73KO Light/Dark Box

Mice were placed in the light/dark box for 5 min with access between the light and dark compartments. The amount of time each mouse spent in the light box was recorded. CD73KO (KO, n=18) mice showed a trend towards spending more time in the light box than wild type mice (WT, n=18), but this was not statistically significant. Data are means ± SEM.

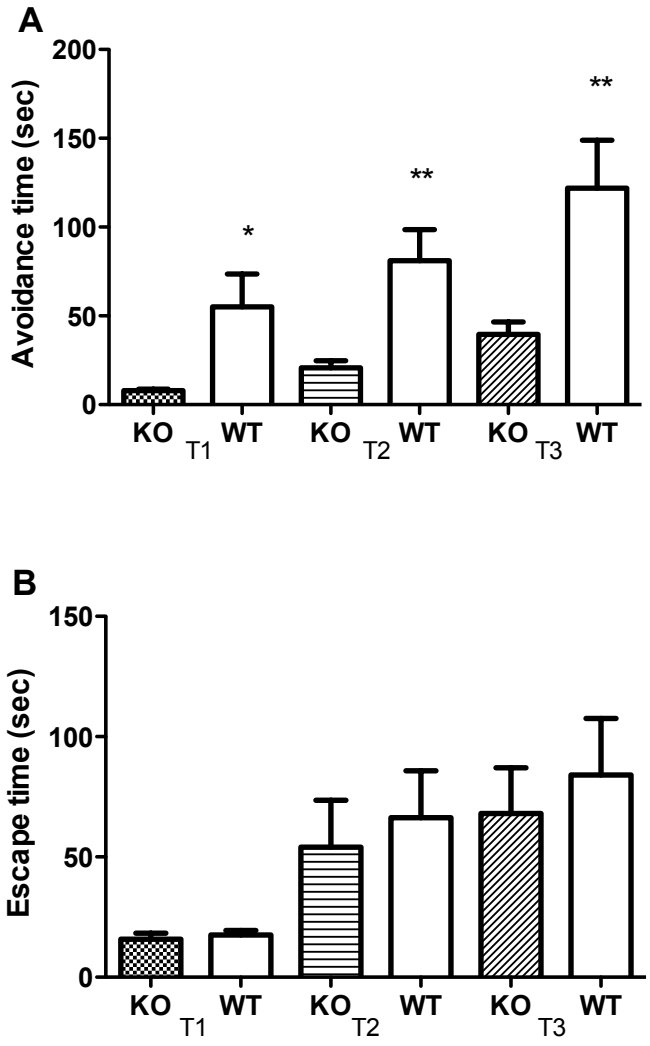


Figure 22. CD73KO Elevated T Maze Data

CD73KO and wild type mice were put on the ends of either the closed or the open arm of the ETM three times. Latency for each mouse to first get out of the closed arm was recorded as the avoidance time, and the latency for each mouse to first get back to the closed arm from the open arm was recorded as escape time. (A) A significant reduction of all three avoidance times was found with the CD73KO (KO, n=18) mice compared with wild type mice (WT, n=18); (B) No significant differences were found in the escape times between genotypes. Data are means ± SEM, $^{*}p<0.05$, $^{**}p<0.01$, two tailed, unpaired t test.

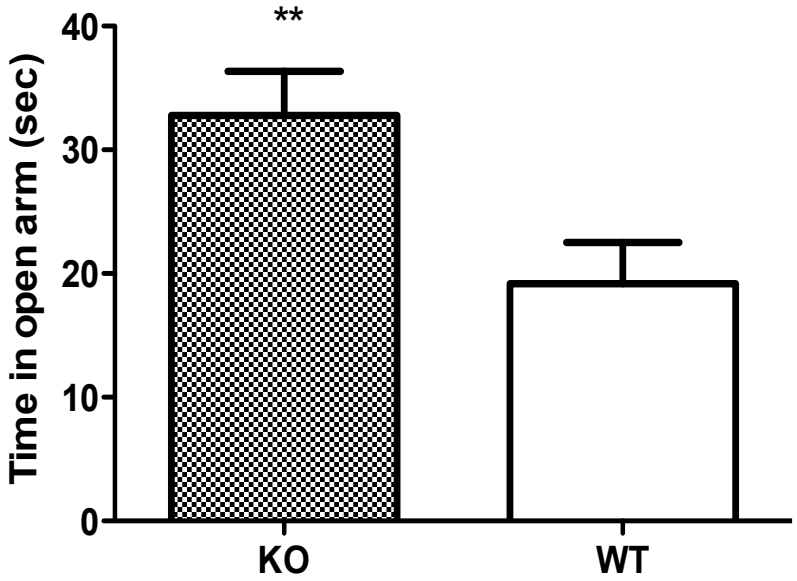


Figure 23 Elevated Plus Maze Data

CD73KO and wild type mice were put on the central square of the EPM facing the closed arm, and their activities were monitored for 5 min. The amount of time that each mouse spent in the open arm was recorded. The CD73KO mice showed reduced anxiety-like behaviour on elevated plus maze test. The CD73KO mice (KO, n=18) spent a significantly greater amount of time in the open arm compared with wild type mice (WT, n=18). Data are means ± SEM, $^{**}p<0.01$, t test.

Chapter 5. DISCUSSION

The main findings of this thesis were (1) the increase of hENT1 expression increased with gene dosage; (2) the behavioural responses of hENT1 transgenic mice to ethanol and caffeine were altered relative to their wild type littermates but the extent of alteration was not affected by gene dosage; and (3) CD73 knockout mice showed increased LORR and reduced anxiety-like behaviours than wild type C57Bl6 mice.

Adenosine is a substance ubiquitously found in all cells. Through activating its four subtypes of G protein coupled receptors, which are integral plasma membrane proteins termed A_1, A_{2A}, A_{2B}, and A_3, adenosine plays important roles in many physiological functions (Fredholm *et al.*, 2001). Adenosine modulates neuronal excitability, locomotor activity, arousal and neuroinflammation (Dunwiddie *et al.*, 2001). Also, adenosine is important in many neurological disorders, such Parkinson's and Huntington's disease (Fredholm *et al.*, 2005).

Adenosine is a neuromodulator that is formed both intracellularly and extracellularly. In the intracellular pathway, adenosine is produced from ATP and is transported through cell membranes via nucleoside transporters, such as ENT1. Also, adenosine can be produced extracellularly from ATP, or other adenine nucleotides released from cells by an ecto-enzyme pathway that includes CD73. These extracellular nucleotides can signal through purinergic receptor families P2X and P2Y (Fredholm *et al.*, 2005). The regulation of adenosine levels in the extracellular space is key to regulating the activity of adenosine receptors. A better understanding of the source of extracellular adenosine could give us targets that drugs can bind to alter the adenosine level in the extracellular space. Thus, better treatments could be developed for one or more psychiatric or neurological disorders or diseases.

In this study, we have used two different types of mice with genetic modifications, hENT1 transgenic mice and CD73 knockout mice. We used these mice to address the relative importance of extracellular and intracellular pathways in physiological conditions or after stimulation with pharmacological tools or pathological conditions.

In the present study, we confirmed that the expression of the hENT1 transporters increased with the increase of the gene dosage. [^{3}H]NBMPR is a high affinity and selective radioligand for ENT1. [^{3}H]NBMPR binding assay was performed to measure the abundance of total ENT1 (mouse ENT1 and hENT1) in mice. An approximate 4.5 fold increase was detected in the heterozygous transgenic mice relative to their wild type littermates and a further 2.5 fold increase was found in homozygous mice. Since the radioligand binding experiments confirmed that heterozygous and homozygous transgenic mice have overexpression of the ENT1 transporter, we performed experiments to investigate how overexpression of ENT1 affects mice. These experiments may indicate the role of adenosine in basal and experimental conditions. However, the results may be due to changes in adenosine levels with changes in adenosine receptor activation. The results may also be due to, or affected by, compensatory changes in adenosine receptor abundance. [^{3}H]DPCPX is a selective radioligand for A_1 receptors and [^{3}H]ZM 241385 is a selective radioligand for A_{2A} receptors (Fredholm *et al.*, 2001). Results from our binding assays suggest that there is no difference in A_1 and A_{2A} receptor levels between transgenic and wild type littermate mice. The results indicated that no difference in adenosine A_1 and A_{2A} receptor levels was caused by the overexpression of hENT1.

Ethanol and caffeine are two important lifestyle drugs that act through adenosinergic mechanisms. The relationship between adenosine receptors and ethanol's pharmacological

actions is not clearly understood. Ethanol affects many different cell functions. It has effects on α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) (Martin *et al.*, 1995), N-methyl-D-aspartate (NMDA), and γ-amino butyric acid (GABA) receptors (Ikonomidou, 2000), Na^+/K^+ ATPase (Kashkin *et al.*, 2002), and intracellular purine metabolism (Szabo *et al.*, 1999). Acute administration of ethanol has been found to increase extracellular adenosine (Gordon *et al.*, 1986), which may be due to increased formation of adenosine (Carmichael *et al.*, 1991) or to inhibition of ENT1 (Krauss *et al.*, 1993; Nagy, 1992). Studies by other investigators showed a reduced LORR duration in ENT1 null mice (Choi *et al.*, 2004). These findings are consistent with the results presented here that show that hENT1 transgenic mice are more sensitive to ethanol than their wild type littermates. Furthermore, the ethanol – induced ataxia can be enhanced by adenosine A_1 receptor agonists and decreased by antagonists (Dar, 1990; Dar, 2001). Caffeine is a non-selective competitive adenosine receptor antagonist, which has similar affinity for A_1 and A_{2A} receptors (Fredholm, 1995a). The action of caffeine depends upon the presence of agonist activated adenosine receptors. Our previous study (Parkinson *et al.*, 2009) showed a decrease in caffeine sensitivity in heterozygous transgenic mice relative to their wild type littermates, which may indicate a decrease in extracellular adenosine levels as reported in a separate study from our lab (Zhang *et al.*, 2011).

Behavioural tests were performed with hENT1 transgenic mice and the results confirmed the findings of previous studies from our lab (Parkinson *et al.*, 2009). From the results of the wheel running test and locomotor activity experiment, we observed a normal activity level in transgenic mice similar to their wild type littermates. In ethanol and caffeine experiments, hENT1 transgenic mice showed significant differences relative to their wild type littermates. However, the results did not show significant statistical difference between

heterozygous and homozygous transgenic mice. This may be because the difference in ENT1 abundance is smaller between homozygous and heterozygous transgenic mice (2.5 fold) than it is between heterozygous and wild type mice (4.5 fold).

In our LORR experiments, the results have showed a greater hypnotic effect produced by ethanol in transgenic mice than their wild type littermates and a trend towards an increased effect on homozygous relative to heterozygous mice but this was not statistically significant.

CPA is an adenosine selective A_1 receptor agonist. Studies have shown that adenosine receptor agonists have sedating effects (Jain *et al.*, 1995). The present results also showed that the activity level of mice was greatly reduced after the injection of CPA. No significant differences were observed between wild type and transgenic mice, which was consistent with the lack of a difference in A_1R density observed with radioligand binding assays using [^{3}H]DPCPX.

Results from ethanol and caffeine experiments confirmed our previous findings (Parkinson *et al.*, 2009). Heterozygous and homozygous transgenic mice showed increased ethanol sensitivity and decreased caffeine sensitivity, relative to wild type mice. No statistically significant differences were observed between homozygous and heterozygous transgenic mice. Due to the competitive antagonism of caffeine at adenosine receptors, a decreased stimulatory effect of caffeine may indicate a reduced level of adenosine in the extracellular level.

CD73 knockout mice were obtained and tested with a series of behavioural experiments. CD73 catalyzes the last step in extracellular adenosine formation. The depletion of CD73 abolishes the extracellular pathway of adenosine formation, and thus adenosine levels in the extracellular space are mainly from other sources, such as cellular release via

nucleoside transporters.

Behavioural experiments with ethanol injections showed increased hypnotic effect and decreased ethanol induced ataxia effect in CD73 knockout mice relative to wild type mice. In the LORR experiment, an increased ethanol hypnotic effect was observed in CD73 knockout mice relative to wild type mice at high dose (3.6g/kg). Because ethanol can inhibit ENT1, the level of adenosine transport through this nucleoside transporter should be reduced, thus, an increased hypnotic effect suggests that the extracellular pathway for adenosine formation predominates. However, this would not be expected in CD73 knockout mice. In the rotarod experiment, decreased ethanol induced ataxia was observed in CD73 knockout mice relative to wild type mice at lower doses (1g/kg and 1.5g/kg ethanol); this effect is consistent with reduced adenosine levels and adenosine receptor activity. Further experiments are required to explain the increased LORR and decreased ataxia effect in CD73 KO mice.

In the caffeine sensitivity experiments, CD73 knockout mice showed a significantly higher activity level compared to wild type mice in the acclimatization period (first 2 h in chamber before injection). However, no difference was observed after the injection of caffeine. These data indicated a slower habituation to the novel environment by CD73 KO mice and was investigated further with additional tests of anxiety-like behaviours.

Previous studies showed that adenosine analogues are anxiolytics (Dunwiddie *et al.*, 2001). Also, adenosine A_1 receptor knockout mice show signs of increased anxiety (Fredholm *et al.*, 2005). Furthermore, the blockade of $A_{2A}R$ by caffeine at higher doses can elicit anxiety (Fredholm *et al.*, 1999), including panic attacks in mice, and $A_{2A}R$ null mice exhibit increased aggressiveness and anxiety-like behaviours compared to their wild type counterparts (Ledent *et al.*, 1997). Therefore, a series of anxiety tests were performed on CD73 knockout mice.

These anxiety tests involve many different anxiogenic stimuli, so we can observe anxiety-like behaviours. The locomotor experiment showed similar activity levels in CD73 knockout mice and wild type mice. The open field experiment is used as measure of anxiety-like behaviour. Mice tend to avoid highly illuminated, novel, open spaces (Crawley *et al.*, 1980), so the open field acts as an anxiogenic stimulus and allows for measurements of anxiety induced locomotor activity and exploratory behaviours. CD73 knockout mice stayed longer in the inner zone of the open box. This indicates that CD73 knockout mice were less likely to avoid the anxiogenic stimuli compared to wild type mice.

Light/dark box experiment has been widely used to assess anxiety-like behaviour in rodents, with the bright light and white box being potentially more anxiogenic than the darker box. The results showed that CD73 knockout mice tended to stay a longer time in the lighted areas, but this was not statistically significant.

Studies suggested that conditioned fear is associated with generalized anxiety disorder and unconditioned fear is related to panic disorder (Deakin *et al.*, 1991). The ETM was adopted on the assumption that avoidance is related to conditioned fear, whereas one-way escape is related to unconditioned fear (Carvalho-Netto *et al.*, 2004). CD73KO mice showed reduced anxiety-like behaviour, as the avoidance time was significantly reduced, relative to wild type mice. No difference was observed in escape times. These results suggest that CD73KO mice have reduced anxiety-related behaviours, but no changes in panic-related defensive behaviours.

The EPM test involves conflicts between the tendency to explore and the tendency to avoid the anxiogenic stimuli of open and high spaces in mice (Pellow *et al.*, 1985). In this

experiment, CD73 knockout mice stayed a significantly greater amount of time in the open arm. This indicates a lower anxiety level in CD73 knockout mice relative to wild type mice.

From the results of anxiety tests, CD73 knockout mice showed less anxiety behaviours compared to wild type mice. This may indicate a greater amount of adenosine in the extracellular space in CD73 knockout mice than wild type mice. But further evidence is needed, such as the use of adenosine receptor antagonists.

In summary, from the present study, hENT1 transgenic and CD73 knockout mice showed altered behavioural response to drugs that act directly or indirectly on adenosine receptors. CD73 knockout mice indicated reduced anxiety-like behaviour. It may become a future target for the treatment of anxiety. Behavioural experiments often produce more variation compared to *in vitro* studies. However, it is very important to study the animals as a live unit. In order to give a more confident conclusion, different behavioural experiments need to be performed. Sometimes experimental data may generate large variations, which obscure the difference between genotypes. In order to have better results from the behavioural experiments, investigators need to be consistent with all the factors that may affect the result, including the experiment procedure, same investigator to manipulate, room temperature, and even the eye contact may be produce a variation in the experiments. In present study, changes in behaviour suggested changes in adenosine levels and adenosine receptor activity.

Genetically modified mice are useful tools to study the regulation of adenosine pathways. However, changes in adenosine concentration still remain to be studied. In order to have more understanding of the relationship between the extracellular and intracellular pathway, further studies are needed.

In the future, further studies could be done. First, we can plan experiments that test the

correlations between ENT and CD73 pathways. We can block the CD73 pathway in hENT1 transgenic mice, in order to block one critical source of adenosine outside the cell. We can also study the expression and abundance of ENT1 in CD73 knockout mice. Compensatory changes in this transport may be involved after changes in extracellular adenosine formation. Second, measurement of adenosine concentrations is very important. Using techniques such as microdialysis, we can target specific brain regions and collect cerebrospinal fluid (CSF) to measure adenosine concentrations in normal state and concentrations after introduction of ethanol. Third, we can infuse adenosine to these genetically modified animals and measure their adenosine receptor activity. Moreover, additional characterization of these genetically modified mice in other behaviours can be done in the future. Adenosine is also involved in behaviours such as sleeping behaviour and pain nociception, more characterization may lead us to a better understanding of the adenosine regulation.

Chapter 6. REFERENCES

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