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EXTRACELLULAR ADENOSINE AS A NOVEL NEUROIMMUNE MODULATOR

BY

GABRIEL SEAN CHIU

DISSETATION

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Doctoral Committee:

Professor Rodney W. Johnson, Chair
Professor Gregory G. Freund, Director of Research
Professor Jeffrey A. Woods
Assistant Professor Ryan N. Dilger
ABSTRACT

Adenosine is a pleotropic purine nucleoside with actions ranging from neurotransmission to immunomodulation. Pathologic processes that trigger cellular stress and damage also cause significant increases in local and systemic concentrations of adenosine. Coinciding with elevation in adenosine is an increase in brain-based IL-1. Perturbed cognition appears to be a sequela of acute hypoxia but the mechanisms by which cognitive functions are impacted are not clearly defined. In this study, we first examined the impact of acute hypoxia on learning and memory and its relationship to the inflammasome. Mice were exposed to normobaric 6% oxygen/94% nitrogen for 2 hours (hypoxia) or ambient air (normoxia). Mice required 6 hrs of reoxygenation before they could be successfully trained in novel object recognition post-hypoxia. Mice also lost the ability to be fear conditioned if trained post-hypoxia. When mice were administered interleukin-1 receptor antagonist prior to hypoxia, mice could be successfully trained in novel object recognition 4 hrs post-hypoxia. Post hypoxia learning also recovered in 4 hrs in IL-1 receptor 1 knock-out mice. Importantly, when the caspase-1 inhibitor, YVAD-CMK, was administered by intracerebroventricular (i.c.v.) injection into the brain, post-hypoxia learning recovered in 4 hrs. Similarly, the administration of the adenosine receptor (AR) antagonist, caffeine, expedited recovery from anterograde amnesia. Furthermore, hypoxia/reoxygenation more than doubled brain caspase 1 activity, while adenosine alone was able to elicit a similar response. This reoxygenation-dependent activation of caspase 1 was prevented by administration of either caffeine or by targeted antagonism of A1/A2A ARs. These findings suggest that anterograde amnesia after hypoxia/reoxygenation is reliant, in part, on IL-1β generated by AR-dependent activation of caspase 1 after reoxygenation.
Next, we set out to investigate the signaling pathway in which adenosine can trigger caspase-1 activation. Here, we show that mice perfused with 50 μm adenosine demonstrate a brain-based increase in IL-1β and caspase-1 activity coupled to increases in intracellular cAMP and PKA activity. Co-perfusion of adenosine with the adenosine A2A receptor antagonist, 8-(3-Chlorostyryl)-caffeine, prevented adenosine-dependent activation of caspase-1 as did use of adenosine A2A receptor knockout (KO) mice. Co-perfusion of adenosine with the K_{ATP} channel blocker, glyburide, also blocked adenosine-dependent activation of caspase-1. Finally, mice i.p. injected with 50 μm adenosine displayed reduced locomotor activity and food intake and increase anxiety-like bahviors. These adenosine-dependent behaviors were prevented in caspase-1, IL-1R1 and adenosine A2A receptor KO mice. Furthermore, when mice were injected with the caspase-1 inhibitor, Ac-YVAD-CMK, i.c.v., they were protected against a peripheral injection of adenosine, suggesting that brain-based IL-1β induction is required after adenosine administration. Taken together, our data indicate that adenosine & the adenosine A2A receptor promotes immobility, anorexia, and anxiety via PKA-mediated K_{ATP} channel opening and subsequent K+ efflux-dependent activation of the inflammasome that is unique to the brain.

Lastly, we tested the interaction between hypoxia/reoxygenation and adenosine dependent anxiety-like behaviors. Similar to before, mice were exposed to 6% oxygen for 2 hours and allowed to recover. We see that hypoxia/reoxygenation elicited over 2.5-fold increase in circulating adenosine levels, which returned to normal 2 hours after hypoxia. This increase was mirrored with an elevation in active caspase-1 staining in neurons of the amygdala but not the hippocampus, suggesting a region specific upregulation. Furthermore, we see that hypoxia/reoxygenation and subsequent increase in circulating adenosine was anxiogenic in WT animals, while A2A AR KO animals were protected, further suggesting the role of adenosine in
hypoxia/reoxygenation-induced anxiety-like behaviors. Taken together, the data suggests that hypoxia/reoxygenation induces an upregulation in neuron-based caspase-1 via A2A AR signaling in the amygdala, which subsequently can cause amnesia and anxiety.
Dedicated to my family: my mother, father and brother.

Thank you for your encouragements and love. You have supported me in all of my life’s decisions and taught me the importance of hard work. I will carry your lessons with me forever.
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CHAPTER 1: LITERATURE REVIEW

Abstract

Adenosine is a pleiotropic neuromodulator that acts as a signal during states of energy imbalance. It functions through four (A1, A2A, A2B, A3) cell surface G protein-coupled adenosine receptors (AR) to inhibit (A1 and A3) or stimulate (A2A and A2B) adenylyl cyclase and subsequently change cAMP concentration. Physiological concentration of extracellular adenosine exerts inhibitory effects on neurons and decrease neurotransmission as a possible neuroprotective mechanism to prevent overexcitation. However, many disease states have been associated with an elevated concentration of adenosine that can activate pathological pathways where prolong inflammation and cellular damage can occur. The duality of action yielded many hypotheses of possible mechanisms involved in adenosine signaling. This review will explore the various influences adenosine has on many systemic and neurological diseases and propose possible novel therapies.

Introduction

Adenosine is an endogenous purine nucleoside that has been shown to work through four G protein-coupled receptors; where two (A1 and A3) are shown to inhibit and two activate (A2A and A2B) adenylyl cyclase to cause changes in cAMP concentration. Under physiological conditions, adenosine, as a metabolic intermediary of ATP breakdown, serves as a neuromodulator (Fredholm et al., 1994). Adenosine exhibits various tonic effects throughout the body, however, this review will be focused on diseases pertaining energy imbalance and the neurological disorders, where it is tied to functions including sleep (Portas et al., 1997), arousal
(Lin et al., 1997) and memory (Pereira et al., 2005). Extracellular concentrations fluctuate, yielding highest during wakefulness (30 nM) than during sleep (24 nM) (Porkka-Heiskanen, 1997, Huston et al., 1996). However, during metabolic and cellular stress caused by inflammatory responses, extracellular ATP, ADP, and AMP can be quickly phosphohydrolyzed to adenosine in the interstitial space by ecto-5’-nucleotidase, also known as CD73 (Hart et al., 2008; Kobie et al., 2006; Riksen et al., 2006). Here, we review the function of extracellular adenosine and discuss the potential impact on diseases and possible interventions through the paradoxical adenosine signaling.

**Purine and adenosine metabolism**

Extracellular purine nucleotides and nucleosides can act as signaling molecules (Ralevic & Burnstock, 1998; Zimmermann. 2000). Metabolism of these purines is presumed in all tissues and most cell types to inactivate these signals via cell-surface enzymes. Fig. 1.1 summarized the mechanism of purine degradation in the intra- and extracellular fraction. Due to the size and charge of ATP, it cannot diffuse through cell membranes and require precise regulation on release via cytolysis, vesicular release, or activation of transport proteins. Intracellular ATP concentration is usually around 1 mM while extracellular concentration remains comparatively low (µM concentration) (Corriden & Insel, 2010). On the contrary, adenosine concentration remains relatively balanced across the membrane (between 20-30 nM) (Porkka-Heiskanen, 1997, Huston et al., 1996) due to greater permeability via adenosine transporters (Deussen et al., 1999) and production can be attributed to both intra- and extracellular metabolism of ATP.

The hydrolysis of ATP and ADP to AMP is primarily catalyzed by ATP-diphosphohydrolase, also known as apyrase, with extracellular $K_m$ values between 10-20 µM.
(Zimmermann, 1996), while specific ATP- and ADPases and ecto-nucleotide pyrophosphatases may also produce AMP. Furthermore, formation of ATP and AMP from ADP is also possible via ATP:AMP phosphotransferase (reviewed in Zimmermann, 1996). On the other hand, the majority of adenosine is generated by a single family of enzyme, 5’-nucleotidases, with the best classified being the ecto-5’-nucleotidase which is responsible for the degradation of AMP in the interstitial space. Extracellularly, ecto-5’-nucleotidase exhibit an affinity to AMP in the µM concentration. Intracellularly, 5’-nucleotidases has a high $K_m$ for AMP in the mM range with subsequent bi-directional transport of adenosine across the membrane (Zimmermann, 1996; Zimmermann & Braun, 1996; Latini & Pedata, 2001). Lastly, it is important to note that intracellular production of adenosine is also possible from hydrolysis of S-adenosylhomocysteine, however, unlike production via AMP, this pathway is not linked to the energy state of the cell and does not appear to produce significantly in the brain (Pak et al., 1994; Latini et al., 1996). Furthermore, it appears that ecto-5’-nucleotidase is mainly expressed, via immunocytochemical analysis, on the membranes of astrocytes, oligodendrocytes, and microglia (Schoen et al., 1987; Grondal et al., 1988) suggesting an important role of adenosine in immune response. Interestingly, it is also important to note that ecto-5’-nucleotidase is also expressed on neurons exclusively in the rodent hippocampus (Zimmermann, 1996; Zimmermann & Braun, 1996;; Schoen et al., 1999), presumably acting as a regulatory signal for excitability (Boison, 2007; Dunwiddie & Masino, 2001, Dunwiddie et al., 1997). Taken together, the data suggests that extracellular ATP is quickly broken down to adenosine as a possible immune regulator and a counter-regulation for excitotoxicity in the hippocampus.

The irreversible deamination of adenosine to inosine in the interstitial space is catalyzed by adenosine deaminase (ADA). In humans, polymorphism leading to reduced expression of
Adenosine deaminase is the cause of a form of severe combined immunodeficiency (ADA-SCID) (Sanchez et al., 2007). Symptoms of ADA-SCID include chronic inflammatory diseases (Blackburn & Kellems, 2005) and toxicity to T and B cells (Kantoff et al., 1986) further suggesting the importance of adenosine in regulating immune responses.

**Adenosine signaling**

Adenosine signals via four membrane bound G protein-coupled adenosine receptors (AR), divided into A1, A2A, A2B, and A3 subclasses of the P1 purine receptor family (Fredholm et al., 2001). All AR recognize extracellular adenosine and are antagonized by caffeine (Fredholm et al., 1999). Adenosine exerts its effects by regulating cAMP concentration. At physiological, sub-µM concentrations, the A1 receptor decreases intracellular cAMP via the Gi inhibition of adenylyl cyclase. Similar to the A1 receptor, the A3 receptor is also coupled to Gi to decrease cAMP concentration (Fig.1.1). However, it is in a relatively low abundance and appears to have minimal role in the brain (Rebola et al., 2005). During pathological states where extracellular adenosine concentration rise above µM range, the A2A receptor activates adenylyl cyclase to increase intracellular cAMP (Calker et al., 1979). Another receptor in the subclass of A2 receptor, the A2B receptor can also stimulate adenylyl cyclase at a much lower affinity to adenosine than A2A (Hide et al., 1992; Liang & Haltiwanger, 1995).
Adenosine in diseases

Being the backbone of both ATP and cAMP, adenosine is tied to the metabolic state of a cell. An increase in extracellular adenosine concentration is seen when energy expenditure is increased (Porkka-Heiskanen & Kalinchuk, 2010). Similarly, adenosine can also regulate various metabolic pathways including glucose metabolism (Nemeth et al., 2007; Dong et al., 2008) and inflammatory responses (Blackburn, 2003). Streptozotocin-induced hyperglycemia, a model of type-1 diabetes, is attenuated by A1 but not A2A AR agonism (Nemeth et al., 2007), where overexpression of A1AR in mice appears to protect mice from diet-induced insulin resistance (Dong et al., 2008) and activation of the A1 receptor inhibits lypolysis via inhibition of adenylyl cyclase (Dhalla et al., 2009). Conversely, the activation of adenylyl cyclase via A2A stimulation appears to promote glycogenolysis (Magistretti et al., 1986). Taken together, it appears that inhibition of adenylyl cyclase via adenosine signaling appears to promote insulin signaling. Furthermore, it appears that adenosine levels elevate in response to obesity (Kaartinen et al., 1991) with a paralleled increase in circulating insulin levels (Xu et al., 2003), further suggesting a link between adenosine and insulin signaling. Indeed, inhibition of adenylyl cyclase adenosine potentiates insulin-induced activation of PI3K. Lastly, it is important to note that the diabetic-like state enhances adenosine-evoked inhibition of hippocampal neurons (Morrison et al., 1992).

Cognition

The physiological activation of the A1AR during high energy consumption is thought to decrease neuronal function by hyperpolarizing neurons and prevent neurotransmitter release, mainly glutamate, dopamine, serotonin, and acetylcholine (Boison, 2007; Cunha, 2005; Porkka-
Extracellular adenosine concentration is shown to increase during neuronal firing and is co-released with certain neurotransmitters (Dulla & Masino, 2013). It is suggested that the ability of adenosine to downregulate the brain during intense energy expenditure is an auto-protective role to minimize cell damage, while the diurnal fluctuation of adenosine concentration suggests that it may also have a role in regulating sleep and wakefulness (Porkka-Heiskanen & Kalinchuk, 2010; Porkka-Heiskanen, 1997, Huston et al., 1996). A rise in extracellular adenosine, however, has been linked to pathological conditions via the A2AAR, where inhibitors of the receptor have been shown to be neuroprotective (Cunha, 2005). It is important to note that both the A1 and A2A receptors can form complexes with neurotransmitter receptors to exert further regulation on neuronal transmission (Cunha, 2005). Table 1 summarizes a partial list of the known interactions between adenosine and certain neurological diseases (Boison, 2008).

**Conclusion**

The studies summarized in this review support the hypothesis that a dichotomous relationship exists between physiological and pathological adenosine receptor signaling. The normal functions of adenosine via A1 receptors appear to regulate sleep and wakefulness (Portas et al., 1997). As a signal or cellular energy balance, adenosine can be released in response to neuronal firing (Dulla & Masino, 2013) as a means to silence neurons. A similar mechanism exists to prevent overstimulation in neurological diseases such as in epilepsy, ischemia, or schizophrenia (Boison, 2006). Therefore, A1-specific agonism may be beneficial in treating overactivity in the brain. However, when extracellular adenosine concentration reaches µM
levels, such as in pathological states of hypoxia or infection, the activation of the A2A receptor appears to elicit detrimental effects (Abbrachio & Cattabeni, 2006; Fredholm, 2010). Inhibition of the A2A receptor appears to be beneficial in prolonged diseases such as Alzheimer’s and Parkinson’s diseases (Dall’Ihna et al., 2003; Jacobson & Gao, 2006). This may be relevant in disease prevention when introducing the ubiquitous AR inhibitor, caffeine, to regular therapy (Fredholm, 2010; Rosso et al., 2008; Ascherio et al., 2001). Due to the opposing forces on adenylyl cyclase and cAMP, it appears that the bolstering of A1 signaling and the blocking of A2A signaling may confer synergistic benefits in neurological disorders (Gomes et al., 2011). In summary, the current research shows that augmented adenosine signaling appears to be involved in many neurological diseases. Therefore, specific, targeted agonism and antagonism of adenosine receptors may be novel and emerging targets.
Abstract

After hypoxia, a critical adverse outcome is the inability to create new memories. How anterograde amnesia develops or resolves remains elusive, but a link to brain-based IL-1 is suggested due to the vital role of IL-1 in both learning and brain injury. We examined memory formation in mice exposed to acute hypoxia. After reoxygenation, memory recall recovered faster than memory formation impacting novel object recognition and cued fear conditioning but not spatially cued Y-maze performance. The ability of mice to form new memories after hypoxia/reoxygenation was accelerated in IL-1 receptor 1 knockout (IL-1R1 KO) mice, in mice receiving IL-1 receptor antagonist (IL-1RA) and in mice given the caspase 1 inhibitor, Ac-YVAD-CMK. Mechanistically, hypoxia/reoxygenation more than doubled caspase 1 activity in the brain which was localized to the amygdala compared to the hippocampus. This reoxygenation-dependent activation of caspase 1 was prevented by broad-spectrum adenosine receptor (AR) antagonism with caffeine and by targeted A1/A2A AR antagonism with 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine. Additionally, perfusion of adenosine activated caspase 1 in the brain while caffeine blocked this action by adenosine. Finally, resolution of anterograde amnesia was improved by both caffeine and by targeted A1/A2A AR antagonism. These findings indicate that amygdala-based anterograde amnesia after
hypoxia/reoxygenation is sustained by IL-1β generated through adenosine-dependent activation of caspase 1 after reoxygenation.

**Introduction**

Injury to the brain through loss of oxygen triggers memory loss and causes learning deficiencies (Shukitt-Hale et al., 1996) including anterograde amnesia (Beatty et al., 1987). Importantly, acute hypoxia activates the neuroimmune system especially its IL-1 arm (Johnson et al., 2007). Brain-based IL-1 regulates cognitive function (Dantzer et al., 2008) and excess IL-1 in the brain is congruous with memory loss and impaired learning (Pugh et al., 2001). While neuroimmune system-generated IL-1 can cause brain injury (Ma et al., 2003), the mechanism by which IL-1 is produced in the brain during reduced oxygen conditions is not understood. IL-1α and IL-1β are both present in the brain and each is implicated in complications related to hypoxia and ischemia (Touzani et al., 1999). Previously, we demonstrated that delayed recovery from acute hypoxia, as measured by social withdrawal in mice, was reliant on IL-1β because inhibition of the inflammatory caspase, caspase 1, dramatically shortened recuperation time (Johnson et al., 2007). As a member of the cysteine-aspartic acid protease family, caspase 1 exists intracellularly as an inactive proenzyme (Damiano et al., 2004) until it is proteolytically processed by Nod-like receptor (NLR)-containing multi-protein inflammasomes (Miao et al., 2011). Activated caspase 1 enzymatically processes pro-IL-1β to a secretable mature form (Bauernfeind et al., 2009). Inflammasome activation is elicited by a variety of microbe- and host-associated bioactives (Schroder et al., 2010) including endogenous danger signals generated during reoxygenation like reactive oxygen species (ROS) (Tschopp et al., 2010), uric acid (Lamkanfi et al., 2007) and ATP (Di Virgilio, 2007).
The restoration of oxygen after hypoxia is required for recovery but can, itself, cause tissue damage (González-Correa et al., 2007). Reoxygenation is frequently described in conjunction with reperfusion as occurs in ischemic injuries like myocardial infarction (Galaris et al., 1989) and stroke (Kostulas et al., 1999). Recently, reoxygenation unassociated with reperfusion has been linked to neural injury and cognitive dysfunction associated with sleep apnea (Gozal et al., 2001). Hypoxia/reoxygenation can precipitate endoplasmic reticulum (ER) stress (Bi et al., 2005), cell death (Saikumar et al., 1998) and inflammation (Johnson et al., 2007) but the means by which hypoxia/reoxygenation triggers these squelae is not clear. Currently, hypoxia/reoxygenation-dependent generation of ROS is a favored causative to reoxygenation injury (Li et al., 2002) but membrane destabilization is also a consequence of hypoxia/reoxygenation (Bickler et al., 1994; Calabresi et al., 1995). Importantly, membrane damage causes increases in extracellular concentrations of ATP, ADP and adenosine (Guinzberg et al., 2006).

Intracellular adenosine concentrations rapidly increase during states of negative energy balance when ATP hydrolysis outstrips ATP synthesis (Bruns, 1991; Fredholm et al., 1999). In contrast, extracellular adenosine is primarily derived from enzymatic phosphohydrolysis of ATP in the interstitial space (Hart et al., 2008). During hypoxia and ischemia, the extracellular concentration of adenosine can increase from 30-300 nM (Rudolphi et al., 1997) to 10-50 µM (Hagberg et al., 1987). In addition, extracellular AMP derived from intracellular ATP and ADP can be phosphohydrolyzed by CD73 to adenosine (Kobie et al., 2006). G protein-coupled adenosine receptors (ARs), which are divided into the subclasses A1, A2A, A2B and A3, all
recognize extracellular adenosine (Fredholm et al., 2001) and are blocked by the non-selective lipophilic competitive antagonist and nutraceutical, caffeine (Fredholm et al., 1999), as well as specific pharmacologic inhibitors. Therefore, in this study we sought to show that memory formation after acute hypoxia is adversely impacted by brain IL-1β through a mechanism reliant on AR-dependent activation of caspase 1.

**Methods**

**Materials-** All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) except as noted. All primers were purchased from Applied Biosystems (Foster City, CA).

**Animals-** Animal use was conducted in accordance with Institutional Animal Care and Use Committee approved protocols at the University of Illinois. C57BL/6J male animals were purchased from Jackson Laboratory (Bar Harbor, ME) at 7 weeks of age. C57BL/6J IL-1R1 knock-out (IL-1R1 KO) mice were bred in-house. Mice were group housed (×8 cage) in standard shoebox cages (length 46.9 cm; width 25.4 cm; height 12.5 cm) and allowed water and food ad libitum. Housing temperature (72 °F) and humidity (45–55%) were controlled as was a 12/12 h reversed dark-light cycle (2200–1000 h). Video recording of animal behavior was performed under red light using a Night Shot capable video camera (Sony HDR-XR500V). Except for locomotor activity which was performed as a repeated measure, all treatments at all time points represent separate cohorts of mice. The total number of mice utilized was 630.

**ICV cannulation-** As we have previously described (Johnson et al., 2007), mice were anesthetized IP with a sodium ketamine hydrochloride/xylazine hydrochloride solution
delivering 80 mg/kg ketamine and 12 mg/kg xylazine. Animals were placed in a Kopf stereotaxic instrument (David Kopf Instruments, Tujunga, CA) and mouse-specific brain infusion cannulas (Plastics One, Roanoke, Va) placed using the coordinates 0.6 mm posterior, 1.5 mm lateral to the bregma and 2.5 mm ventral from the surface of the skull. Cannulas were fixed to the skull with cyanoacrylate gel adhesive (Plastics One) and protected by a plastic guard. Mice were allowed 7 days to recover.

*Hypoxia/Reoxygenation*- As we have previously described (Sherry et al., 2009), mice (n = 16/episode) were transferred from their home cages to a BioSperix ProOx/A-Chamber Biological Atmosphere System (Biospherix, Lacona NY) and subjected to either a 6% oxygen + 94% nitrogen environment (hypoxia) or an atmospheric air environment (normoxia) for 2 h. After exposure mice were returned to their home cages.

*Injectables*- As we have previously described (Johnson et al., 2007), the caspase 1 inhibitor, Ac-YVAD-CMK, (Bachem, Torrance, CA) was administered ICV at a dose of 50 ng/μL/mouse immediately prior to hypoxia. Kineret (recombinant IL-1 RA) (AmGen, Thousand Oaks, CA) was administered IP at a dose of 1.4 mg/kg/mouse 30 min prior to hypoxia. CafCit (caffeine citrate) (Bedford Laboratories, Bedford, OH) was administered IP at a dose of 100 mg/kg/mouse immediately prior to hypoxia. 8-cyclopentyl-1, 3-dipropylxanthine (A1 AR antagonist, 2.5 mg/kg/mouse) + 3, 7-dimethyl-1-propargylxanthine (A2A AR antagonist, 2.5 mg/kg/mouse) (Chen et al., 2001) were administered as an IP cocktail 1 hour prior to hypoxia. N-Acetyl Cysteine (NAC) (Hospira, Inc., Lake Forest, IL) was administered IP at a dose of 50 mg/kg/mouse immediately prior to hypoxia. For all studies, vehicle/control injection was
phosphate buffered saline (PBS) or normal saline (saline), as indicated, except for targeted AR inhibition which was 1:5 DMSO/castor oil.

**Novel object recognition**- Novel object recognition was performed as we have previously described (Lavin et al, 2011, York et al., 2011). In studies examining retrograde amnesia, mice (1 h prior to hypoxia) were individually transferred from their home cage to a home cage-sized memory arena containing two identical objects placed 10 cm apart at the short-side wall end for 5 min (training). Mice were then subjected to hypoxia or normoxia. At the time points indicated post-hypoxia, mice (individually) were transferred back to the memory arena now containing one familiar object and one unfamiliar object (novel object) (testing). Investigative behavior of the objects was video recorded for 5 min and evaluated using EthoVision XT 7 (Noldus Information Technology) video tracking software. Percent investigation was calculated by dividing the time spent investigating each object by the total time spent investigating both objects. In studies examining anterograde amnesia, mice were transferred for training at the time points indicated post hypoxia. Mice were then returned to their home cage for 55 min. As above, testing was initiated by returning mice to the memory arena with one familiar object and one novel object. Investigative behavior of the objects and time spent examining each object was performed as above.

**Locomotion**- Spontaneous locomotor activity was measured as we have previously described (Lavin et al., 2011, York et al., 2012). At the times indicated, mice were video recorded in their home cage for 5 min. Distance moved was quantified using EthoVision XT 7.
Cued fear conditioning- Cued fear conditioning was performed as we have previously described (York et al., 2011). 4 h post hypoxia, mice were placed in a Lafayette Instruments Cued and Contextual Fear Test Chamber (Lafayette Instruments, Lafayette IN). After a 30 sec acclimation period, mice were exposed to a white light (~23000 lux) for 2 sec, followed by a 2 sec foot shock (60 V, 1 mA). After a 30 sec wait, mice were re-exposed to the light + foot shock cycle as above (training). Mice were then returned to their home cage. At the time points indicated, mice were reintroduced to the testing apparatus and allowed to acclimate for 30 sec. Mice then underwent two cycles of light without foot shock similar in parameters to the above (testing). All freezing behavior was evaluated via an integrated infrared photo beam array. Data were analyzed using the MotorMonitor Host Software (Lafayette Instruments).

Alternation- Spatially cued spontaneous alternations was performed as we have previously described (Lavin et al, 2011, York et al., 2011). In brief, mice were placed in a symmetrical 3-arm, clear Plexiglas Y-maze (40 cm length x 9 cm width x 16 cm height per arm with an arm angle of 120⁰) with side walls decorated with black triangles, black circles or black diagonal lines. Mice were randomly placed in one of the arms. Movement was recorded for 5 min and mouse exploration evaluated from the video record. Mice were tested at 4, 52 and 76 hours after hypoxia. Results are presented as the ratio of perfect alternations to total arm entrances. Perfect alternations were defined as exploration of two novel arms sequentially prior to a return to the start arm independent of a right or left arm choice at initiation. To have entered an arm, the mouse was required to have all four legs in that arm.
**Quantitative PCR (qPCR)**- As we have previously described (York et al., 2012), RNA was isolated from the hippocampus dissected from PBS perfused whole brains. RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (PN 4368813) (Applied Biosystems). The TaqMan Gene Expression primers used were: Glial fibrillary acidic protein (GFAP) (Mm01253033_m1), aquaporin 4 (Mm000802131_m1), CD11b (Mm00434455_m1), F4/80 (Mm00802529_m1) and the peripheral benzodiazepine receptor (PBR) (Mm00437828_m1). qPCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). To compare gene expression, a parallel amplification of endogenous RPS3 (Mm00656272_m1) was performed. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene to RPS3 was performed by comparing ΔCts, where Ct is the threshold concentration.

**Caspase 1 activity**- PBS perfused whole brains, brain regions (as indicated) and livers were frozen in liquid nitrogen then freeze fractured in reaction buffer containing 50 mM NaCl (Fisher Scientific, Fair Lawn, NJ), 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM bestatin, 1 mM pepstatin (EMD4Bioscience, Darmstadt, Germany), 1 mM 4-((2-aminoethyl)benzenesulfonyl fluoride hydrochloride and 50 mM HEPES, pH 7.4 (USB Corporation, Cleveland, OH) using the TissueLyser II (Qiagen, Valencia, CA) at a rotational frequency of 30/sec for 2 min. Lysates were clarified at 16,000 x g for 15 min at 4º C and the supernatant protein concentrations determined using the DC Protein Assay (Bio-Rad, Hercules, CA) and a ELx800 Absorbance Microplate Reader (BioTek Instrument, Winooski, VT). Supernatant protein concentrations were normalized to 10 mg/ml (whole brain and liver) or 2.5 mg/ml (brain regions) with reaction
buffer. Caspase 1 activity was determined colorimetrically in the clarified lysates using the caspase 1 substrate Ac-YVAD-p-nitroaniline (p-NA) (Enzo Life Science, Farmingdale, NY) at a final concentration of 4 mM. Substrate incubation was at 37º C for the times indicated. Moles of p-NA liberated was determined by a standard curve ranging from 0.075 mM to 0.3 mM p-NA (Enzo Life Science, Farmingdale, NY). Caspase 1 activity was calculated as (Δ[p-NA]/Δ time)/(total protein).

**Adenosine perfusion**: Mice were euthanized via CO₂ asphyxiation and the heart immediately exposed using straight 11.5 cm scissors (FST, Foster City, CA). The left ventricle was pierced with a 23 gage 1.25 inch needle (BD, Franklin Lakes, NJ) attached to a BD 30 mL syringe. Mice were perfused (as indicated) with 10, 30, 50 or 100 µM adenosine, 500 µM NAC, 500 µM caffeine, 50 µM adenosine + 500 µM caffeine or 50 µM adenosine + 500 µM NAC in PBS, pH of 7.4 or PBS, pH 7.4 alone.

**Glutathione (GSH)**: Similar to methods we have previously described (Godbout et al., 2002), GSH and glutathione disulfide (GSSG) were measured using the Glutathione Assay Kit (Sigma-Aldrich). As above, PBS perfused whole brains were frozen in liquid nitrogen then freeze fractured in the kit provided Assay Buffer using the TissueLyser II. After brain homogenization, GSH/GSSG was quantified spectrophotometrically following the instructions of the manufacturer and a ELx800Absorbance Microplate Reader

**Phospho (p)-ERK 1/2, p-p38 MAPK and p-JNK**: Similar to methods we have previously described (Sherry et al., 2007), whole brains were frozen in liquid nitrogen then freeze fractured,
as above, in a homogenization buffer containing 50 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM sodium orthovanadate, 250 nM okadaic acid, 1:200 Protease Inhibitor Cocktail III (Calbiochem, Darmstadt, Germany) and 50 mM HEPES, pH 7.4 using the TissueLyser II. Lysates were clarified at 16,000 x g for 15 min at 4º C and the supernatant protein concentrations determined using the DC Protein Assay, as above. p-ERK 1/2, p-p38 MAPK and p-JNK were measured in 50 µL of lysate using a Bio-Plex phosphoprotein assay (Bio-Rad, Hercules, CA) and Luminex 100 System (Luminex, Austin, TX) following the instructions of the manufacturer. Results are expressed relative change in phosphorylation/total protein.

**Immunohistochemistry**- Similar to methods we have previously described (Johnson et al., 2007, Davis-Devine et al., 2003), mice were perfused with ice-cold 10% neutral buffered formalin. Brains were removed and using a Mouse Brain Slicer (Zivic Instruments, Pittsburgh, PA) coronal sections ranging from the bregma to -3.0 mm from the bregma where generated. These slices were fixed in 10% neutral buffered formalin for 24 hr then paraffin embedded and sectioned. A 4 µm section at -1.7 mm from the bregma was immunostained for GFAP using a rabbit anti-GFAP antibody (DAKO, Carpinteria, CA) at a dilution of 1:2000 at RT for 30 min. Detection was performed using the Rabbit Link/SS Label detection kit (Biogenex, San Ramon, CA) in conjunction with the Biogenex i6000 Automated Staining System (incubation time was 15 min). After coverslipping, the entire slide was imaged at 40x with a NanoZoomer 2.0-HT (Hamamatsu, Bridgewater, NJ).

**Uric Acid**- Blood was collected via cardiac puncture using a 26G x 3/8 inch needle (Becton Dickinson, Franklin Lakes, NJ) and allowed to stand at room temperature for 30 min.
Serum was generated by centrifuging samples at 10,000 x g for 15 min. Serum uric acid was determined on a AU680 analyzer (Beckman Coulter, Brea CA).

Statistics- All data are presented as mean ± SEM. Data were analyzed using Sigma Plot 11.2 (Systat Software, Chicago, IL). To test for statistical differences, a one-way or two-way ANOVA was used with or without repeated measurements where needed. Tukey’s test was used for post-hoc pair-wise multiple comparison procedures. Where indicated, raw data was transformed using a log10 transformation to attain equal variance. All statistical analysis included testing for time point x treatment interactions. Statistical significance was denoted as p < 0.05.

Results

Restoration of memory recall after acute hypoxia- Fig.2.1A demonstrates that if memory formation (training) occurred 1 h prior to hypoxia, the ability of mice to recall that memory in the testing phase was restored after 4 h of reoxygenation (82.5 ± 2.1% vs 81.3 ± 2.1%, normoxia vs hypoxia). Immediately after hypoxia mice did not explore either a familiar or novel object. After 1, 2 and 3 h of reoxygenation mice explored a novel object as if it were a familiar object (51.8 ± 5.7%, 61.6 ± 6.2% and 72.6 ± 3.6%, respectively). Main effects of hypoxia (p < 0.001) and time (p < 0.001); 0 h time point: p < 0.001, normoxic v hypoxic (82.5 ± 1.9% v 0 ± 0%); 1 h time point: p < 0.001, normoxic v hypoxic (79.2 ± 1.6% v 51.8 ± 5.7%); 2 h time point: p < 0.001, normoxic v hypoxic (81.9 ± 2.1% v 61.6 ± 6.2%); 3 h time point: p < 0.05, normoxic v hypoxic (84.5 ± 2.4% v 72.6 ± 3.6%). After 4, 5 and 6 h of reoxygenation mice preferably explored the novel object over the familiar (81.3% vs 18.7%, 77.5% vs 22.5% and 75.7% vs
24.3%, respectively). Fig. 2.1B shows that hypoxic mouse spontaneous locomotor activity was comparable to that of normoxic mice after 2 hrs of reoxygenation. Main effects of hypoxia (p < 0.05) and time (p < 0.05); 0 h time point: p < 0.001, normoxic v hypoxic (1910.4 ± 128.9 cm v 314.7 ± 159.1 cm); 1 h time point: p < 0.05, normoxic v hypoxic (1772.5 ± 128.5 cm v 1297.7 ± 115.3 cm).

Memory formation recovers more slowly than memory recall after acute hypoxia-

Fig. 2.2A illustrates that if memory formation occurred after hypoxia (training) the ability of mice to learn does not recover until 6 h of reoxygenation (66.2 ± 1.0% vs 76.3 ± 1.8%). At 3, 4 and 5 h of reoxygenation, mice exposed to hypoxia explored a novel object 54.3 ± 0.8%, 57.0 ± 2.4% and 55.9 ± 1.5%, respectively. Main effects of groups (p < 0.001); normoxic v 3 h time point: p < 0.001 (75.3 ± 1.9% v 54.3 ± 0.8%), normoxic v 4 h time point: p < 0.001 (75.3 ± 1.9% v 57.0 ± 2.47%), normoxic v 5 h time point: p < 0.001 (75.3 ± 1.9% v 55.9 ± 1.5%). Data was transformed. Fig. 2.2B confirms that memory formation after hypoxia is impaired. In the cued fear conditioning test, both normoxic and hypoxic mice (after 4 hrs of reoxygenation) had similar immobility when first exposed to cue/foot shock (training). When retested with just the cue (testing), after 5 and 52 hrs of reoxygenation, normoxic mice demonstrated a 121.9% and 123.8% increase in immobility, respectively, when compared to the initial cue/foot shock exposure (training). In contrast, hypoxic mice when retested with just the cue at 5 and 52 hrs of reoxygenation showed immobility comparable to the initial cue/foot shock exposure (training). Main effects of hypoxia (p < 0.001) and time (p < 0.05); 4 h time point: p < 0.659, normoxic v hypoxic (83.0 ± 3.7 sec v 80.7 ± 4.4 sec); 5 h time point: p < 0.05, normoxic v hypoxic (101.1 ± 3.3 sec v 84.8 ± 3.1 sec); 52 h time point: p < 0.05, normoxic v hypoxic (102.7 ± 3.3 sec v 86.2 ±
3.6 sec). Fig. 2.2C shows that perfect alternations in a cued Y-maze were not affected by hypoxia at 4, 52, and 76 hours after reoxygenation. Main effects of hypoxia (p = 0.626) and time (p = 0.235).

**Knockout of IL-1R1 improves memory formation and locomotion while blunting activation of ERK1/2 and p38 MAPK** - Fig. 2.3A demonstrates that if memory formation (training) occurred 4 hrs after hypoxia, IL-1R1 KO mice had accelerated recovery of memory formation. At 5 hrs of reoxygenation, hypoxic IL-1R1 KO mice explored a novel object similar to normoxic wild type (WT) mice (70.2 ± 7.4% vs 63.5 ± 3.1%). Hypoxic WT mice explored a novel object as if it were a familiar object (47.9 ± 4.7%). Main effects of genotype (p < 0.05) and hypoxia (p = 0.235); normoxic WT v hypoxic WT: p < 0.05 (63.5 ± 3.1% v 47.9 ± 4.7%), normoxic IL-1R1 KO v hypoxic IL-1R1 KO: p = 0.645 (66.9 ± 3.3% v 70.2 ± 7.4%), hypoxic WT v hypoxic IL-1R1 KO: p < 0.05 (47.9 ± 4.7% v 70.2 ± 7.4%). Similar results were seen in mice administered IL-1 RA (Fig. 2.3B) (main effects of treatment (p < 0.05) and hypoxia (p = 0.055); saline normoxic v saline hypoxic: p < 0.05 (66.9 ± 5.3% v 53.2 ± 5.3%), IL-1RA normoxic v IL-1RA hypoxic: p = 0.457 (72.1 ± 3.4% v 67.2 ± 4.0%), saline hypoxic v IL-1RA hypoxic: p < 0.05 (53.2 ± 5.3% v 67.2 ± 4.0%)) and in mice administered Ac-YVAD-CMK (Fig. 2.3C) (main effects of treatment (p < 0.05) and hypoxia (p = 0.5); PBS normoxic v PBS hypoxic: p < 0.05 (69.7 ± 3.5% v 55.7 ± 4.2%), Ac-YVAD normoxic v Ac-YVAD hypoxic: p = 0.157 (67.1 ± 3.3% v 75.5 ± 5.0%), PBS hypoxic v Ac-YVAD hypoxic: p < 0.05 (55.7 ± 4.2% v 75.5 ± 5.0%)). Fig. 2.3D illustrated that spontaneous locomotor activity of IL-1R1 KO mice was restored after 1 hr of reoxygenation as opposed to 2 hrs in hypoxic WT mice. Main effects of hypoxia (p < 0.001), genotype (p = 0.781) and time (p = 0.193); 0 h time point: normoxic WT v
AR blockade prevents hypoxia-dependent activation of caspase 1 in the brain- Fig.2.4A shows that after 1 hr of reoxygenation, brain caspase 1 is 208.8% more active in hypoxic mice than normoxic mice. Hypoxic mice not allowed to significantly reoxygenate did not demonstrate an increase in brain caspase 1 activity. In addition, after 6 hrs of reoxygenation, brain caspase 1 activity was similar to that of normoxic mice. 0 h time point: p = 0.525, normoxic v hypoxic (1.0 ± 0.1 fold v 1.1 ± 0.2 fold); 1 h time point: p < 0.001, normoxic v hypoxic (1.0 ± 0.2 fold v 2.1 ± 0.2 fold); 6 h time point: p = 0.930, normoxic v hypoxic (1.0 ± 0.1 fold v 1.0 ± 0.1 fold). Figs.4B&C illustrate that mice pre-treated with either caffeine or an A1/A2A AR inhibitor cocktail did not up-regulate caspase 1 activity in the brain after 1 hr of reoxygenation (saline
hypoxic v caffeine hypoxic: p < 0.001 (2.6 ± 0.0 fold v 1.0 ± 0.2 fold); vehicle hypoxic v A1/A2A antag hypoxic: p < 0.05 (2.6 ± 0.3 fold v 1.5 ± 0.1 fold)). Table 1 demonstrates that after 1 hr of reoxygenation hypoxia lowers the brain GSH/GSSG ratio by 49% (p < 0.05) and that caffeine and NAC each prevented this decline. The GSH/GSSG ratio was unchanged if hypoxic mice were not afforded significant time to reoxygenate (1.00 ± 0.22 fold vs 1.02 ± 0.22 fold, normoxia vs hypoxia; p = 0.832). Importantly, NAC pre-treated mice did not lose the ability to up-regulate brain caspase 1 activity after reoxygenation (Fig.2.4D). Main effects of treatment (p = 0.358) and hypoxia (p < 0.001); saline normoxic v saline hypoxic: p < 0.05 (1.0 ± 0.0 fold v 2.1 ± 0.5 fold), NAC normoxic v NAC hypoxic: p < 0.05 (1.24 ± 0.2 fold v 2.2 ± 0.4 fold), saline hypoxic v NAC hypoxic: p = 0.498 (2.1 ± 0.5 fold v 2.2 ± 0.4 fold). Fig.2.4E shows that mice perfused with 30, 50 and 100 µM adenosine have a 167%, 225% and 247% increase in brain caspase 1 activity, respectively. PBS perfused v adenosine perfused. Main effect of treatment (p < 0.001). 0 µM v 10 µM: p = 0.844 (1.0 ± 0.1 v 0.8 ± 0.1), 0 µM v 30 µM: p < 0.05 (1.0 ± 0.1 v 1.6 ± 0.2), 0 µM v 50 µM: p < 0.001(1.0 ± 0.1 v 2.3 ± 0.3), 0 µM v 100 µM: p < 0.001(1.0 ± 0.1 v 2.5 ± 0.1), 10 µM v 30 µM: p = 0.01 (0.8 ± 0.1 v 1.6 ± 0.2), 10 µM v 50 µM: p < 0.001(0.8 ± 0.1 v 2.3 ± 0.3), 10 µM v 100 µM: p < 0.001(0.8 ± 0.1 v 2.5 ± 0.1), 30 µM v 50 µM: p = 0.075 (1.6 ± 0.2 v 2.3 ± 0.3), 30 µM v 100 µM: p = 0.023 (1.6 ± 0.2 v 2.5 ± 0.1), 50 µM v 100 µM: p = 0.940 (2.3 ± 0.3 v 2.5 ± 0.1). Figs.4F&G demonstrate that caffeine but not NAC inhibits adenosine-dependent activation of caspase 1 when adenosine is perfused into mice (Fig.2.4F, main effect of treatment (p < 0.001). PBS v adenosine: p < 0.001 (1.0 ± 0.1 v 1.9 ± 0.1), adenosine v caffeine: p < 0.001 (1.9 ± 0.1 v 0.9 ± 0.1), adenosine v adenosine + caffeine: p < 0.001 (1.9 ± 0.1 v 1.3 ± 0.1), adenosine + caffeine v caffeine: p = 0.06 (0.9 ± 0.1 v 1.3 ± 0.1), PBS v adenosine + caffeine: p = 0.267 (1.0 ± 0.1 v 1.3 ± 0.1), PBS v caffeine: p = 0.841 (1.0 ±
0.1 v 0.9 ± 0.1). Fig.2.4G, main effect of treatment (p < 0.05). PBS v adenosine: p < 0.05 (1.0 ± 0.0 v 1.9 ± 0.1), adenosine v NAC: p < 0.05 (1.9 ± 0.1 v 1.2 ± 0.3), adenosine v adenosine + NAC: p = 0.991 (1.9 ± 0.1 v 1.8 ± 0.1), adenosine + NAC v NAC: p = 0.076 (1.8 ± 0.1 v 1.2 ± 0.3), PBS v adenosine + NAC: p < 0.05 (1.0 ± 0.0 v 1.8 ± 0.1), PBS v NAC: p = 0.897 (1.0 ± 0.0 v 1.2 ± 0.3)). Finally, to examine another potential activator of the inflammasome, uric acid was examined. After 1 hr of reoxygenation serum uric acid levels were similar in normoxic and hypoxic mice (3.3 ± 0.4 mg/dL vs 3.1 ± 0.2 mg/dL).

*Hypoxia induces brain region-specific activation of caspase 1*- Fig.2.5A shows that 1 hr after hypoxia/reoxygenation, caspase 1 activity in the amygdala compared to control was increased 180% (normoxic v hypoxic, 1.0 ± 0.1 v 1.8 ± 0.1, p < 0.001). Hippocampal caspase 1 activity was not impacted by hypoxia/reoxygenation (normoxic v hypoxic: 1.0 ± 0.1 v 0.7 ± 0.1, p = 0.134) nor was pre-frontal cortex or cerebellar caspase 1 activity (data not shown). To determine if significant gliosis occurred after hypoxia, immunohistochemistry for GFAP was performed. Fig.2.5B demonstrates no change in GFAP expression at 1 and 6 hrs of reoxygenation in both WT and IL-1R1 KO mice. Additionally, qPCR for GFAP and aquaporin 4 gene transcripts were examined in the amygdala and hippocampus at 1 and 6 hrs after reoxygenation in WT and IL-1R1 KO mice revealing no impact of hypoxia (data not shown). To examine microglia infiltration/proliferation/activation, gene transcripts for CD11b, F4/80 and PBR were examined in the amygdala and hippocampus after 1 and 6 hr of reoxygenation in WT and IL-1R1 KO mice. No differences were observed (data not shown).
AR blockade speeds recovery of memory formation after hypoxia - Fig. 2.6A demonstrates that if memory formation (training) occurred 4 hrs after hypoxia, mice administered caffeine had accelerated recovery of memory formation. At 5 hrs of reoxygenation, hypoxic mice pre-treated with caffeine explored a novel object similar to normoxic mice (68.0 ± 3.6% vs 69.5 ± 5.6%). Hypoxic mice pre-administered vehicle explored a novel object as if it were a familiar object (48.6 ± 2.4%). Main effects of treatment (p < 0.05) and hypoxia (p < 0.05); saline normoxic v saline hypoxic: p < 0.05 (69.5 ± 5.6% v 48.6 ± 2.4%), caffeine normoxic v caffeine hypoxic: p = 0.838 (69.2 ± 4.6% v 68.0 ± 3.6%), saline hypoxic v caffeine hypoxic: p < 0.05 (48.6 ± 2.4% v 68.0 ± 3.6%). Fig. 2.6B demonstrates that if memory formation (training) occurred 4 hrs after hypoxia, mice administered 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-proparglyxanthine had accelerated recovery of memory formation. At 5 hrs of reoxygenation, hypoxic mice pre-treated with 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-proparglyxanthine explored a novel object similar to normoxic mice (76.1 ± 3.7% vs 73.5 ± 3.4%). Hypoxic mice pre-administered vehicle explored a novel object as if it were a familiar object (62.4 ± 3.2%). Main effects of treatment (p = 0.055) and hypoxia (p = 0.197), hypoxia x treatment (p < 0.05); vehicle normoxic v vehicle hypoxic: p < 0.05 (73.7 ± 2.7% v 62.4 ± 3.2%), 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine normoxic v 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine hypoxic: p = 0.583 (73.5 ± 3.4% v 76.1 ± 3.7%), vehicle hypoxic v 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine hypoxic: p < 0.05 (62.4 ± 3.2% v 76.1 ± 3.7%).
Discussion

In humans, the causes of a confusional state are numerous and include drugs, toxins, infections, head injuries and metabolic derangements (Gascon & Barlow, 1970, Mori & Yamadori, 1987). Hypoxemia is a well-recognized antecedent to brain injury (Rees et al., 1998) that can precipitate memory loss well beyond hypoxemia-associated confusion or delirium (Berggren, 1987). In rodents, hypoxia has long been known to cause retrograde amnesia (Sara and Lefevre, 1972). The inability of rodents to recall a previously learned task or avoid noxious stimuli after hypoxia can be long-lived (24 h) as Sara et al. show in rats exposed to 3.5% oxygen (Sara and Lefevre, 1972). In addition, this memory loss can occur without significant brain cell death because, in mice, as Kyff et al. show (Kyff et al., 1989) and we confirm, 2 h of hypoxia above 5% oxygen does not lead to identifiable neuronal death even in the hippocampus (Johnson et al., 2007). Here we show that the acute hypoxia used induced minimal neuroinflammation over the 6 hrs examined as reflected by no change in the gene expression of GFAP, aquaporin 4, CD11b, F4/80 and PBR, and no change in GFAP protein expression. These findings indicate that unlike ischemia where all of the aforementioned astrocyte and microglial markers are rapidly up-regulated (Feuerstein et al., 1997, Lu & Sun, 2003, Natale et al., 2003, Taguchi et al., 2007, Taniguchi et al., 2007, Xiong et al., 2009) and significant gliosis occurs, acute hypoxia can be a recoverable event that results in negligible neuropathology.

The mechanism protecting burrowing mammals, especially certain moles which can survive severe oxygen deprivation (3%) for extended times (8 hrs) (Avivi, 2006), from acute hypoxia is not clear but its origin, evolutionarily, appears as a safeguard to burrow collapse. As Fig.2.1A shows, mice exposed to 6% hypoxia for 2 h developed retrograde amnesia that resolved
after 4 hrs of reoxygenation. This loss of memory was not solely due to a motor deficit that prevented mice from performing the task because mice had regained normal locomotor activity after 2 hrs of reoxygenation (Fig.2.1B).

Post hypoxia, the ability of mice to form a new memory did not recover until after 6 h of reoxygenation (Fig.2.2A). Since the training phase for novel object recognition occurred 1 hr prior to the testing phase, memory formation after hypoxia was impaired within the first 5 hrs of reoxygenation. To determine if the results obtained were memory type specific, cued fear conditioning and spatially cued Y-maze performance were utilized as additional tests of memory dysfunction. Fig.2.2B illustrates that with cued fear conditioning mice failed to learn when trained after 4 hrs of reoxygenation and that this was a failure of memory formation and not a deficiency in task performance because even after 52 h of reoxygenation mice did not recall the learned cue. Unlike fear conditioning, mice performance in a spatially cued Y-maze was not impacted by hypoxia (Fig.2.2C) demonstrating spatial memory and hence the hippocampus may be less impacted by hypoxia.

Although hypoxia appears tied to anterograde amnesia in humans (Beatty, 1987), almost no work has been performed in animal models. A single study by Udayabanu et al looked at retrograde and anterograde memory in a mouse model of acute hypobaric hypoxia with a calculated oxygen percentage of 7% (307.4 Torr) for 6 hrs (Udayabanu et al., 2008). They concluded that hypoxia only impacted retrograde memory and not anterograde memory. While they conducted memory formation 2 hrs post hypoxia, the tests they used (passive avoidance step-through and elevated plus maze) were strongly dependent on spatial memory and, thus, the
hippocampus (Yirmiya et al., 2002, Rodgers et al., 1997). Likewise, in our study, spatially cued Y maze performance was not affected by hypoxia while our more amygdala-dependent tests (object recognition and cued fear conditioning) were (Moses et al., 2005). Interestingly, Broadbent et al. showed that in rats spatial memory was impaired when 30-50% of the dorsal hippocampus or 50% of the ventral hippocampus is damaged. Object recognition memory, however, was only impaired after 75-100% of the dorsal hippocampus is injured (Broadbent et al., 2004). Taken together these findings indicate that acute hypoxia impairs memory that is predominantly decoupled from the hippocampus. Support for this contention is seen in Fig. 2.5A which illustrates that hypoxia/reoxygenation increases caspase 1 activation in the amygdala but not in the hippocampus.

Brain IL-1β is important to the recovery of memory formation after hypoxia. Fig. 2.3C shows that the caspase 1 specific inhibitor YVAD-CMK (Wu et al., 2010) administered ICV speeds the recovery of memory formation after hypoxia. Since caspase 1 can process other proteins besides pro-IL-1β (Keller et al., 2008), we confirmed these findings by administering IL-1 RA (Fig. 2.3B) and by using IL-1R1 KO mice (Fig 3A). Since YVAD-CMK was administered ICV, our results indicate that brain generated IL-1β is responsible for impairing memory formation after hypoxia as opposed to IL-1 from the peripheral blood. That a dysregulation in brain IL-1 negatively impacts certain aspects of memory is not surprising. IL-1 is best known for its role in hippocampal-dependent memory (Goshen et al., 2007) and conditions that disrupt IL-1 signaling impair mouse water maze and passive avoidance performance (Yirmiya et al., 2002). As proposed by Goshen and Yirmiya, hippocampal-dependent memory and plasticity are regulated by IL-1 in an inverted U shaped correlation.
where low and high brain IL-1 signaling have similar impacts (Yirmiya et al., 2002). As for the role of IL-1 in novel object recognition, little is known. Costello et al just demonstrated that mice deficient in the single-Ig-interleukin-1 related receptor have impaired novel object recognition as well as an up-regulation of IL-1α but not IL-1β in the brain (Costello et al., 2011). They propose that IL-1α via IL-1R1 may drive certain memory impairments especially as related to infectious etiologies. Fig. 2.5E shows that in hypoxia activation of ERK1/2 and p38 MAPK may be important to IL-1 regulated memory because 1 hr after reoxygenation these kinases had reduced ser/thr phosphorylation in IL-1R1 KO mice when compared to wild type mice. Thus, our results suggest that IL-1β may be more important in disease states involving low-oxygen and that MAPK superfamily members downstream of IL-1R1 may be regulatory.

As we and others have shown, brain IL-1β is elicited during activation of the neuroimmune system (Johnson et al., 2007, Dantzer et al., 2008, Kostulas et al., 1999). Basally, mature IL-1β is nearly undetectable in the rodent brain (Layé et al., 2000; Takao et al., 1993; Taupin et al., 1993) but with neuroimmune stimulation, especially ischemic injury, IL-1β is measurable (Saito et al., 1996) promoting brain injury (Rothwell, 2003). How mature IL-1β is generated in the brain is unknown. Peripherally, the inflammasome is critical to caspase 1 activation and caspase 1 is responsible for the final enzymatic cleavage of pro-IL-1β to secretable IL-1β (Lamkanfi et al., 2007). Fig. 2.4A demonstrates that there is increased brain caspase 1 activity after hypoxia but that reoxygenation is important to this activity because nonreoxygenated mice do no show increased caspase 1 activity. Since reoxygenation appears necessary to brain caspase 1 activation, we examined known danger signals potentially relevant to peripheral inflammasome activation and to hypoxia. ROS are considered to play a role in
reoxygenation/reperfusion injury and redox-dependent activation of the NLRP3 inflammasome has recently been shown (Martinon, 2010; Tschopp & Schroder, 2010). Table 1 demonstrates that hypoxia/reoxygenation markedly reduced the GSH/GSSG ratio indicative of ROS generation. As expected, administration of the GSH precursor and antioxidant, NAC, (Raju et al., 1994) prior to hypoxia prevented a hypoxia/reoxygenation-dependent decline in the GSH/GSSG ratio. Additionally caffeine which has been shown to have antioxidant properties (Devasagayam et al., 1996; Shi & Dalal, 1991) also prevented a decline in the GSH/GSSG ratio after hypoxia/reoxygenation. Unexpectedly, caffeine (Fig. 2.4B) and more importantly antagonism of the A1+A2A ARs (Fig. 2.4C) prevented reoxygenation-dependent activation of caspase 1 while NAC did not (Fig. 2.4D). Furthermore, caffeine (Fig. 2.6A) and A1/A2A AR antagonism (Fig. 2.6B) but not NAC (data not shown) sped recovery from hypoxia-induced loss of memory formation. These findings indicate that adenosine triggers hypoxia/reoxygenation dependent caspase 1 activation resulting in a delay in the ability to form new memories. To further confirm that adenosine activates caspase 1 in the brain, mice were perfused with adenosine. Fig. 2.4E shows that in a dose dependent manner adenosine activates brain caspase 1 and that the EC50 of 30 μM is consistent with interstitial adenosine concentrations seen the microenvironment of hypoxic tissue (10-50 μM) (Sitkovsky et al., 2005). Fig. 2.4F&G demonstrate that caffeine but NAC blocked adenosine-dependent activation of caspase 1 additional illustrating that ARs but not ROSs are important to adenosine-dependent caspase 1 activation.

To date, neither adenosine nor its receptors are implicated as direct activators of the inflammasome or of caspase 1. However, AR antagonism, especially via caffeine, is linked to cognitive improvement in certain neurodegenerative diseases (Cunha et al., 2010) and as a
protectant against Alzheimer’s disease (Cao et al., 2012). How caffeine achieves this function is unclear but it has been postulated that since adenosine acts as an inhibitory neurotransmitter the ability of caffeine to suppress this effect of adenosine is beneficial (Chen et al., 2001). The antioxidant qualities of caffeine have been postulated as a mechanism but this theory does not explain the action of more specific AR antagonists which lack antioxidant properties (ref) and are shown to protect neurons against ischemic injury (Phillis, 1995). Therefore, caffeine acting as an inhibitor of IL-1β generation, as implicated here, is a potentially new mechanism for its action.

How adenosine activates the inflammasome through ARs needs to be defined. Purines are metabolized to uric acid which is a well described activator of the inflammasome (Pétrilli & Martinon, 2007). We measured serum uric acid after hypoxia and saw no increase after 1 h of reoxygenation. This finding is consistent with others who have seen that xanthine oxidase which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid is increased after reoxygenation but only at more distant time points (Cherubini et al., 2000; Gonzalez-Flecha & Cutrin, 1993; Jones et al., 1968; Poulsen et al., 1993). A potential mechanism by which ARs activate caspase 1 is through K+ flux. When triggered by high extracellular concentrations of K+, pannexin 1 channels induce activation of caspase 1 (Silverman et al., 2009). Hypoxia causes increased extracellular concentrations of K+ in brain slice cultures that can be inhibited by AR antagonism (Croning et al., 1995). Importantly, adenosine can stimulate cellular K+ release through the A1 and A2A ARs (Fredholm et al., 2001, Sanjani et al., 2011).
Finally, the brain is rich ARs and they are expressed on a wide range of brain-based cells including microglia (A1, A2A, and A3) (Sperlágh & Illes, 2007), astrocytes (A1, A2A, A2B and A3), neurons (A1 and A2A) (Haskó et al., 2005) and endothelial cells (A2A and A3) (Fredholm et al., 2001; Platts, Duling, 2004). Thus, further work is needed to determine not only how adenosine activates caspase 1 but what are the particular brain cells most responsible for caspase 1 activation and the production of mature IL-1β after hypoxia/reoxygenation. Currently, some specificity to the brain is evident because hypoxia/reoxygenation did not increase liver caspase 1 activity (data not shown). Taken together, our results indicate that hypoxia/reoxygenation increases caspase 1 activity in the brain thereby impairing amygdala-based memory formation. These findings are important because they delineate a new mechanism linking adenosine to activation of the inflammasome.
CHAPTER 3: ADENOSINE THROUGH THE A2A ADENOSINE RECEPTOR
INCREASES IL-1β IN THE BRAIN CONTRIBUTING TO ANXIETY

Abstract

Anxiety is one of the most commonly reported psychiatric conditions, but its pathogenesis is poorly understood. Ailments associated with activation of the innate immune system, however, are increasingly linked to anxiety disorders. In adult male mice, we found that adenosine doubled caspase-1 activity in brain by a pathway reliant on ATP-sensitive potassium (KATP) channels, protein kinase A (PKA) and the A2A adenosine receptor (AR). In addition, adenosine-dependent activation of caspase-1 increased interleukin (IL)-1β in the brain by two-fold. Peripheral administration of adenosine in wild-type animals led to 33 and 42% reduction in spontaneous locomotor activity and food intake, respectively, that were not observed in caspase-1 knockout (KO), IL-1 receptor type 1 (IL-1R1) KO and A2A AR KO mice or in mice administered a caspase-1 inhibitor centrally. Finally, adenosine administration increased anxiety-like behaviors in wild-type mice by 28% in the open field test and by 55% in the elevated zero-maze. Caspase-1 KO, IL-1R1 KO and A2A AR KO mice were resistant to adenosine-induced anxiety-like behaviors. Thus, our results indicate that adenosine can act as an anxiogenic by activating caspase-1 and increasing IL-1β in the brain.

Introduction

With a lifetime prevalence of nearly 18% (Kessler et al., 2005), anxiety disorders including generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic
disorder and post-traumatic stress disorder (PTSD) are among the most common psychiatric conditions suffered by Americans and Europeans (Kessler et al., 2005, WHO 2000). In humans, the pathogenesis of anxiety is poorly understood but recently new triggers of anxiety have been described including oxidative stress (Rammal et al., 2008) and inflammation (Pitsavos et al., 2006).

Common to these intertwined physiologic processes is the generation of the pleotropic cytokine IL-1β (Buttke & Sandstrom, 1994, Donath et al., 2008). IL-1β is not only a regulator of innate immunity, angiogenesis and hematopoiesis but also influences brain-based processes including cognition, locomotion, anorexia and anxiety (Johnson et al., 2007, Dantzer et al., 2008, Pugh et al., 2001, Me et al., 2003). Canonically, IL-1β exists intracellularly as a pro-form that is cleaved to its mature secretable polypeptide by caspase-1 as part of the multiprotein inflammasome (Damiano et al., 2004, Bauernfeind et al., 2009). Signals that activate the inflammasome are varied but potassium (K⁺) efflux is a key event that is especially relevant to the oligomerization and activation of the NLRP3 inflammasome (Pétrilli et al., 2007).

The amygdala is central to emotive learning (Hamann et al., 1999) and appears critical to the aversive state that characterizes anxiety (Davis, 1992). Recently, we demonstrated that the purine nucleoside, adenosine, released from cells during oxidative stress activates caspase-1 in the amygdala. There are four described ARs that respond to extracellular adenosine (Fredholm et al., 2001) with the A2A AR being preferentially expressed in the amygdala when compared to the hippocampus (Rosin et al., 1998). As a stimulatory G protein-coupled receptor (GPCR), the A2A AR is a well described inducer of K⁺ efflux. In the brain, A2A AR-dependent K⁺ efflux has
been linked to ATP-sensitive potassium (K\textsubscript{ATP}) channels downstream of protein kinase A (PKA) (Kleppisch & Nelson, 1995) as part of a hyperpolarization protection mechanism that guards against neuronal excitotoxicity (Popoli et al., 2002). Therefore, this mechanism that helps guard against neuronal damage and death may also be an early activator of the neuroimmune system.

In the brain, adenosine is tied to certain behaviorally-related functions including the regulation of sleep (Portas et al., 1997), arousal (Lin et al., 1997) and memory (Pereira et al., 2005). Normally, the cerebral concentration of extracellular adenosine is highest during wakefulness (30 nM) than during sleep (24 nM) (Porkka-Heiskanen, 1997, Huston et al., 1996). However, in instances of an inflammatory response induced by ischemia, hypoxia and sepsis (Martin et al., 2000, Robertson et al., 2001, Gorlach, 2005), the extracellular concentration of adenosine can easily exceed 50 μM (Hagberg et al., 1987). Therefore, we examined the mechanism by which adenosine activates caspase-1 in the brain and if adenosine triggers anxiety-like behavior via a mechanism reliant on IL-1β.

**Methods**

**Materials**- All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) except as noted. All qPCR assays were purchased from Applied Biosystems (Foster City, CA).

**Animals**- Animal use was conducted in accordance with Institutional Animal Care and Use Committee approved protocols at the University of Illinois. C57BL/6J (C57 WT) and BALB/cJ (BALB/c WT), IL-1R1 knock-out (IL-1R1 KO) on a C57BL/6J background and A2A
AR KO mice on a BALB/cJ background were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Caspase-1 KO (Casp-1 KO) mice on a C57BL/6J background were kindly provided by Dr. Richard Flavell (Yale University School of Medicine, Kuida et al., 1995). Mice were group housed (×8 cage) in standard shoebox cages (length 46.9 cm; width 25.4 cm; height 12.5 cm) and allowed water and food ad libitum. Housing temperature (72 °F) and humidity (45–55%) were controlled, as was a 12/12 h reversed dark-light cycle (2200–1000 h). Video recording of animal behavior was performed under red light using a Sony HDR-XR500V Night Shot capable video camera (Tokyo, Japan). Except for locomotor activity which was performed as a repeated measure, all treatments at all time points represent separate cohorts of mice. Mice were between 8 and 14 weeks of age and the total number of mice utilized was 324.

Perfusions- As we have described (Chiu et al., 2012), mice were euthanized via CO₂ asphyxiation and the left ventricle immediately was pierced with a BD 23 gage 1.25 inch needle (Franklin Lakes, NJ) attached to a BD 30 mL syringe. Mice, as indicated, were perfused with 30 mL of PBS (3 mM KCl, 138 mM NaCl, 8 mM dibasic sodium phosphate, 2 mM monobasic potassium phosphate, pH of 7.4) with or without 50 μM adenosine, 1 μM 8-(3-chlorostyryl)caffeine (CSC), 100 nM KT5720 or 200 μM glyburide. For studies in which mice were perfused with a high concentration of K⁺, PBK (130 mM KCl, 3.5 mM NaCl, 1.5 mM dibasic sodium phosphate, 2 mM monobasic potassium phosphate, pH of 7.4) was substituted for PBS, as indicated.

Caspase-1 activity- As we have described (Chiu et al., 2012), perfused whole brains were frozen in liquid nitrogen then freeze fractured in a reaction buffer containing 50 mM NaCl
(Fisher Scientific, Fair Lawn, NJ), 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM bestatin, 1 mM pepstatin (EMD4Bioscience, Darmstadt, Germany), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and 50 mM HEPES, pH 7.4 (USB Corporation, Cleveland, OH) using a Qiagen TissueLyser II (Valencia, CA). Lysates were clarified at 16,000 x g for 15 min at 4º C and the supernatant protein concentrations determined using the Bio-Rad DC Protein Assay (Hercules, CA) and a BioTek ELx800 Absorbance Microplate Reader (Winooski, VT). Supernatant protein concentrations were normalized to 10 mg/ml with reaction buffer. Caspase-1 activity was determined colorimetrically in the clarified lysates using the caspase-1 substrate Ac-YVAD-p-nitroaniline (p-NA) (Enzo Life Science, Farmingdale, NY) at a final concentration of 4 mM. Substrate incubation was at 37º C for the times indicated. Moles of p-NA liberated was determined by a standard curve ranging from 0.075 mM to 0.3 mM p-NA (Enzo Life Science, Farmingdale, NY). Caspase-1 activity was calculated as (Δ[p-NA]/Δ time)/(total protein). Results are expressed as relative change from control.

PKA activity- Perfused whole brains were frozen in liquid nitrogen then freeze fractured in reaction buffer containing 50 mM NaF, 50 mM β-glycerolphosphate, 2 mM EDTA, 1 mM NaVO₄, 5 mM EGTA, 1 mM DTT, 1 mM benzamidine, 10% glycerol, 1:200 Calbiochem Protease Inhibitor Cocktail III (Darmstadt, Germany), 1 mM phenylmethysulfonyl fluoride (PMSF) and 50 mM HEPES, pH 7.4 using the TissueLyser II. Lysates were clarified and supernatant protein concentrations normalized to 10 mg/ml with reaction buffer, as above. PKA activity was determined using the Enzo Life Science PKA Kinase Activity Kit. In brief, samples were incubated with the kit-provided substrate coated plate for 90 min and phosphorylated
tetramethylbenzidine measured spectrophotometrically at 450 nm. Results are expressed as relative change from control.

**cAMP concentration**- Perfused whole brains were frozen in liquid nitrogen then freeze fractured in reaction buffer containing 138 mM NaCl, 8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 3 mM KCl, 50 mM NaF, 50 mM β-glycerolphosphate, 2 mM EDTA, 1 mM NaVO$_4$, 5 mM EGTA, 1 mM benzamindine, 10% glycerol, 1:200 Calbiochem Protease Inhibitor Cocktail III, 1 mM PMSF, 500 µM 3-isobutyl-1-methylxanthine, 100 µM RO201724 and 50 mM HEPES, pH 7.4 using the TissueLyser II. Lysates were clarified and supernatant protein concentrations normalized to 10 mg/ml with reaction buffer, as above. cAMP concentration was determined using the Promega cAMP-Glo Assay (Madison, WI). In brief, samples were incubated for 30 min and the methyl ester luciferin/luciferase reaction quantified using a Fuji LAS-4000 Imaging System (Tokyo, Japan).

**IL-1β concentration**- As above, perfused whole brains and livers were frozen in liquid nitrogen then freeze fractured in a homogenization buffer containing 50 mM NaCl, 10% glycerol, 1:200 Calbiochem Protease Inhibitor Cocktail III and 50 mM HEPES, pH 7.4 using the TissueLyser II. Lysates were clarified and supernatant protein concentrations measured as above. IL-1β was measured in 50 µL of lysate using the Bio-Rad Bio-Plex Promouse Cytokine Group I Cytokine Assay on a Luminex 100 System (Austin, TX) by methods similar to those we have described (Chiu et al., 2012). Results are expressed as (pg IL-1β)/(μg total protein).
Quantitative PCR (qPCR)- As we have described (Chiu et al., 2012, York et al., 2012), RNA was isolated from perfused whole brains. RNA was reverse transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (PN 4368813). The Applied Biosystems TaqMan Gene Expression primers used were: A2A AR (Mm00802075_m1), IL-1α (Mm99999060_m1), IL-1β (Mm99999061_mH), IL-1 receptor type 1 (IL-1R1) (Mm01226959_m1), IL-1 receptor type 2 (IL-1R2) (Mm00439622_m1), IL-1 receptor antagonist (IL-1RA) (Mm01337566_m1) and caspase-1 (Mm00438023_m1). qPCR was performed on a Applied Biosystems 7900 HT Fast Real-Time PCR System using Applied Biosystems TaqMan Universal PCR Master Mix. To compare gene expression, a parallel amplification of endogenous RPS3 (Mm00656272_m1) was performed. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene to RPS3 was performed by comparing ΔCts, where Ct is the threshold concentration.

Serum adenosine - 500 μL of blood was mixed 1:2 with 138 mM NaCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, 3 mM KCl, 5.9 mM NaHCO₃, 5.6 mM dextrose, 2.15 mM CaCl₂, 1% DMSO, and 120 μM dipyridamole. Samples were centrifuged at 3650 x g for 10 min at 4⁰C and proteins precipitated by addition of 2% v/v of 70% (12N) perchloric acid with re-centrifuged at 3650 x g for 10 min at 4⁰C. Adenosine was determined on a 5500 QTRAP LC/MS/MS (AB Sciex, Foster City, CA) with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA). LC separation was performed on a Phenomenex Kinetex 2.6 u PFP column (4.6 x 100mm) (Torrence, CA). Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. Flow rate was 0.3 mL/min and the linear gradient was: 0-1 min, 100% A; 5 min, 90% A; 10 min,
80% A; 12-18 min, 0% A; and 18.5-25 min, 100% A with an auto-sampler temperature of 5°C. The injection volume was 5 µL. Mass spectra were acquired with positive electrospray ionization with an ion spray voltage of 5500 V at a source temperature of 450 ºC. The curtain gas, ion source gas 1 and ion source gas 2 were 35, 50, and 65, respectively. Multiple reaction monitoring was used to quantify adenosine m/z 268.1 → m/z 136.1, inosine m/z 269.1 → m/z 137.1 and internal standard caffeine m/z 195.1 → m/z 138.1.

Injectables- Adenosine (Akorn, Lake Forest, IL) in PBS was administered IP at a dose of 2 mg/kg/mouse. Ac-YVAD-CMK (Bachem, Torrance, CA) was administered ICV, as we have described (Johnson et al., 2007, Chiu et al., 2012), at a dose of 50 ng/µL/mouse, 30 min prior to IP adenosine injection.

ICV cannulation- As we have described (Johnson et al., 2007, Chiu et al., 2012), mice were anesthetized with an IP injection of sodium ketamine hydrochloride/xylazine hydrochloride solution delivering 80 mg/kg ketamine and 12 mg/kg xylazine. Animals were placed in a David Kopf Instruments stereotaxic instrument (Tujunga, CA), Plastics One mouse-specific brain infusion cannulas (Roanoke, VA) were placed 0.6 mm posterior, 1.5 mm lateral to the bregma and to extend 2.5 mm ventral from the surface of the skull. Cannulas were fixed to the skull with Plastics One cyanoacrylate gel adhesive and protected by a Plastics One guard. Mice were allowed 7 days to recover prior to treatment.

Locomotion- Spontaneous locomotor activity was measured by continuous videography followed by analysis with automated video tracking software (Noldus Information Technology
EthoVision XT 7, Leesburg, VA), as we have described (York et al., 2013). Immediately after treatment, mice were video recorded in their home cage for 60 min. Distance moved was binned into consecutive 15 min intervals. Results are presented as percent control.

*Food intake*- Immediately after adenosine injection, mice were allowed ad libitum access to food in a 6 cm diameter × 1.5 cm glass bowl for 60 min. As we have described (York et al., 2012), food consumption was calculated as the difference in weight of food before and after. Results are presented as percent control.

*Open field test*- Open field testing was performed as we have described (York et al., 2012). In brief, mice were placed in a novel open field arena (66 cm length x 45.7 cm width x 22.9 cm height) 30 min after adenosine injection. Lighting was positioned to create a 9 cm shadow from each side wall. Mice were video recorded for 5 min. Total distance traveled, time spent in the non-shadowed and shadowed areas of the arena were determined using Noldus Information Technology EthoVision XT 7. Percent of time (sec) in the non-shadowed area (center) was calculated by dividing time spent in the center by total time (300 sec) in the arena. Results are presented as percent control.

*Elevated zero-maze*- Elevated zero-maze testing was performed as we have described (31). In brief, 30 min after adenosine injection mice were placed in a circular elevated zero-maze (22.5 cm in diameter, track width of 2.75 cm, elevated 33 cm above the floor). The maze was divided into two open quadrants and two enclosed quadrants. Mice were video recorded for 5 min. Total distance traveled, time spent in the open and closed arms of the maze were
determined using Noldus Information Technology EthoVision XT 7. Percent of time (sec) in the open area was calculated by dividing time spent in the center by total time (300 sec) in the arena. Results are presented as percent control.

Statistics- All data are presented as mean ± SEM. Data was analyzed using Sigma Plot 11.2 (Systat Software, Chicago, IL). To test for statistical differences, a one-way or two-way ANOVA was with repeated measurements where needed. Tukey’s test was used for post-hoc pair-wise multiple comparison procedures. Where indicated, raw data was transformed to attain equal variance. Statistical analysis included testing for time x treatment interactions when needed. Statistical significance was denoted at p < 0.05.

Results

Inhibition of potassium efflux prevents adenosine-dependent activation of caspase-1 in the brain- Fig.3.1A demonstrates that activation of caspase-1 by adenosine is prevented when adenosine is co-perfused with 130 mM K+ (PBK) when compared to 3 mM K+ (PBS). Main effect of interaction, p < 0.001 (PBK vs. adenosine + PBK, 1.07 ± 0.10 vs. 0.93 ± 0.09, p = 0.563: PBS vs. adenosine + PBS, 1.00 ± 0.06 vs. 2.29 ± 0.32, p < 0.001: PBS vs. PBK, 1.00 ± 0.06 vs. 1.07 ± 0.10, p = 0.776: adenosine +PBS vs. adenosine + PBK, 2.29 ± 0.32 vs. 0.93 ± 0.09, p < 0.001). Fig.3.1B shows that activation of caspase-1 by adenosine is blocked when adenosine is co-perfused with the K_{ATP} channel blocker, glyburide. Main effect of adenosine, p = 0.008 (glyburide vs. adenosine + glyburide, 1.06 ± 0.26 vs. 1.29 ± 0.16, p = 0.427: PBS vs. adenosine + PBS, 1.00 ± 0.14 vs. 1.88 ± 0.18, p = 0.003: PBS vs. glyburide (1.00 ± 0.14 vs. 1.06 ± 0.26, p = 0.806: adenosine vs. adenosine + glyburide, 1.88 ± 0.18 vs. 1.29 ± 0.16, p = 0.036).
The PKA inhibitor KT5720 blocks activation of caspase-1 by adenosine- Fig.3.2A shows that activation of caspase-1 by adenosine is blocked when adenosine is co-perfused with KT5720. Main effect of interaction, $p = 0.004$ (KT5720 vs. adenosine + KT5720, $1.07 \pm 0.20$ vs. $1.15 \pm 0.13$, $p = 0.701$; PBS vs. adenosine, $1.00 \pm 0.07$ vs. $2.13 \pm 0.17$, $p < 0.001$; PBS vs. KT5720, $1.00 \pm 0.07$ vs. $1.07 \pm 0.20$, $p = 0.755$; adenosine vs. adenosine + KT5720, $2.13 \pm 0.17$ vs. $1.15 \pm 0.13$, $p < 0.001$). Fig.3.2B&C demonstrate that PKA activity and the concentration of cAMP are increased in the brain after adenosine perfusion, respectively (saline vs. adenosine, $1.00 \pm 0.06$ vs. $1.52 \pm 0.16$, $p = 0.009$; saline vs. adenosine, $1.00 \pm 0.02$ vs. $1.64 \pm 0.19$, $p = 0.015$).

Activation of caspase-1 by adenosine is blocked by selective A2A AR antagonism and in A2A AR KO mice- Fig.3.3A shows that activation of caspase-1 by adenosine is prevented when adenosine is co-perfused with the selective A2A AR antagonist CSC. Main effect of interaction, $p < 0.001$ (CSC vs. adenosine +CSC, $1.23 \pm 0.15$ vs. $1.08 \pm 0.06$, $p = 0.196$; PBS vs. adenosine, $1.00 \pm 0.05$ vs. $1.77 \pm 0.05$, $p < 0.001$; PBS vs. CSC, $1.00 \pm 0.05$ vs. $1.23 \pm 0.15$, $p = 0.053$; Adenosine vs. adenosine + CSC, $1.77 \pm 0.05$ vs. $1.08 \pm 0.06$, $p < 0.001$). Fig.3.3B demonstrates that A2A AR KO mice are protected from adenosine-dependent activation of caspase-1. Main effect of interaction, $p = 0.015$ (A2A AR KO vs. adenosine in A2A AR KO, $0.90 \pm 0.21$ vs. $1.01 \pm 0.19$, $p = 0.610$; WT vs. adenosine in WT, $1.00 \pm 0.08$ vs. $1.83 \pm 0.29$, $p < 0.001$; WT vs. A2A AR KO, $1.00 \pm 0.084$ vs. $0.90 \pm 0.21$, $p = 0.553$; adenosine in WT vs. adenosine in A2A AR KO, $1.83 \pm 0.29$ vs. $1.01 \pm 0.19$, $p = 0.001$).
Adenosine increases IL-1β protein in the brain but not the liver. Table 1 demonstrates that IL-1β protein in the brain, but not the liver, is increased after adenosine perfusion. Adenosine did not induce gene transcripts for IL-1β, IL-1α, IL-1R1, IL-1R2, IL-1RA or caspase-1 (data not shown). Table 2 shows that relative gene expression of A2A ARs is greater in the amygdala than hippocampus.

Adenosine activates caspase-1 in the brain while reducing locomotor activity and food intake. Fig.3.4A demonstrates that brain caspase-1 activity is increased by 33% 15 min after IP injection of adenosine (saline vs. adenosine, 1.00 ± 0.07 vs. 1.33 ± 0.02, p < 0.001).

Concordantly, the serum adenosine concentration was increased by two-fold 15 min after IP injection of adenosine (saline vs. adenosine, 0.399 ± 0.091 μM vs. 0.829 ± 0.051 μM, p = 0.007). Fig.3.4B shows that in C57 WT mice adenosine decreases locomotor activity by 30% 30 through 75 min after injection (saline vs. adenosine: 0-15 min, 100.00 ± 6.24 vs. 98.32 ± 4.33, p = 0.823; 15-30 min, 100.00 ± 9.05 vs. 94.78 ± 8.05, p = 0.669; 30-45 min, 100.00 ± 15.94 vs. 67.48 ± 5.57, p = 0.05; 45-60 min, 100.00 ± 12.00 vs. 69.49 ± 6.97, p = 0.05, 60-75 min, 100.00 ± 8.96 vs. 64.93 ± 5.16, p = 0.006; 75-90 min, 100.00 ± 11.30 vs. 72.75 ± 8.32, p = 0.079; 90-105 min, 100.00 ± 11.76 vs. 112.01 ± 16.92, p = 0.590). Figs. 4C & D show that Casp-1 KO and IL-1R1 KO mice are resistant to adenosine-dependent reductions in locomotor activity (Casp-1 KO) saline vs. adenosine: 0-15 min, 100.00 ± 4.83 vs. 92.98 ± 4.56, p = 0.312; 15-30 min, 100.00 ± 9.00 vs. 109.76 ± 8.78, p = 0.454; 30-45 min, 100.00 ± 8.06 vs. 94.49 ± 13.48, p = 0.719; 45-60 min, 100.00 ± 11.41 vs. 89.95 ± 7.14, p = 0.596; (IL-1R1 KO) saline vs. adenosine: 0-15 min, 100.00 ± 8.64 vs. 113.26 ± 9.22, p = 0.319; 15-30 min, 100.00 ± 17.3 vs. 149.49 ± 21.96, p = 0.108; 30-45 min, 100.00 ± 22.57 vs. 149.48 ± 33.27, p = 0.298; 45-60 min, 100.00 ± 22.64 vs.
Figs.4E & F show, respectively, that in BALB/c WT mice adenosine decreases locomotor activity by 50% 45 min after injection and that A2A AR KO mice are resistant to adenosine-dependent reductions in locomotor activity (BALB/c WT) saline vs. adenosine: 0-15 min, 100.00 ± 20.94 vs. 83.31 ± 13.51, p = 0.455; 15-30 min, 100.00 ± 23.04 vs. 97.53 ± 13.09, p = 0.914; 30-45 min, 100.00 ± 17.70 vs. 105.83 ± 19.92, p = 0.803; 45-60 min, 100.00 ± 13.34 vs. 50.25 ± 18.87, p = 0.015; (A2A AR KO) saline vs. adenosine: 0-15 min, 100.00 ± 14.45 vs. 81.96 ± 10.97, p = 0.420; 15-30 min, 100.00 ± 11.45 vs. 102.00 ± 13.62, p = 0.931; 30-45 min, 100.00 ± 10.77 vs. 92.27 ± 15.19, p = 0.741; 45-60 min, 100.00 ± 4.41 vs. 95.48 ± 10.79, p = 0.808. Figs.4G & H demonstrate that Casp-1 KO mice, IL-1R1 KO mice and A2A AR KO mice are resistant to adenosine-dependent reductions in food intake (C57 WT) saline vs. adenosine, 100.00 ± 10.91 vs. 58.33 ± 10.11, p = 0.006; (Casp-1 KO) saline vs. adenosine, 100.00 ± 9.45 vs. 150.00 ± 18.26, p = 0.029; (IL-1R1 KO) saline vs. adenosine, 100.00 ± 12.60 vs. 86.67 ± 15.07, p = 0.499; (BALB/c WT) saline vs. adenosine, 100.00 ± 12.48 vs. 61.14 ± 7.87, p = 0.006; (A2A AR KO) saline vs. adenosine, 100.00 ± 6.79 vs. 102.08 ± 9.13, p = 0.875.

Central administration of the caspase-1 inhibitor Ac-YVAD-CMK blocks adenosine-induced reductions in locomotor activity and food intake - Figs.5A & B show that C57 WT administered Ac-YVAD-CMK ICV are protected from adenosine-induced decrease in locomotor activity and food intake, respectively (locomotion) saline vs. adenosine: 0-15 min, 100.00 ± 10.92 vs. 61.14 ± 2.61, p = 0.013; 15-30 min, 100.00 ± 45.41 vs. 73.64 ± 5.48, p = 0.468; 30-45 min, 100.00 ± 31.94 vs. 36.08 ± 7.75, p = 0.009; 45-60 min, 100.00 ± 21.41 vs. 40.32 ± 5.42, p = 0.018; Ac-YVAD-CMK vs. adenosine: 0-15 min, 100.00 ± 13.50 vs. 74.39 ± 6.53, p = 0.080; 15-
30 min, 100.00 ± 24.51 vs. 69.02 ± 10.93, p = 0.395; 30-45 min, 100.00 ± 12.09 vs. 93.17 ± 7.40, p = 0.868; 45-60 min, 100.00 ± 18.50 vs. 137.96 ± 14.10, p = 0.109; (food intake) saline vs. adenosine, 100.00 ± 9.62 vs. 53.33 ± 9.72, p = 0.012; Ac-YVAD-CMK vs. adenosine, 100.00 ± 21.25 vs. 104.17 ± 26.90, p = 0.905.

Adenosine causes anxiety-like behavior - Fig.3.6A shows that mice administered adenosine 30 min prior to open field testing exhibited a 28% decrease in percentage of time spent in the center area while Casp-1 KO, IL-1R1 KO and A2A AR KO mice were resistant (C57 WT) saline vs. adenosine: 100.00 ± 7.46% vs. 72.45 ± 6.86%, p = 0.02; (Casp-1 KO) 100.00 ± 27.61% vs. 88.75 ± 14.63%, p = 0.714; (IL-1R1 KO) saline vs. adenosine: 100.00 ± 18.56% vs. 88.24 ± 11.91%, p = 0.646; (BALB/c WT) saline vs. adenosine: 100.00 ± 3.81% vs. 66.75 ± 7.47%, p = 0.007; (A2A AR KO) saline vs. adenosine: 100.00 ± 14.36% vs. 108.82 ± 10.43%, p = 0.630. Fig.3.6B demonstrates that adenosine did not curtail mouse locomotion within the open field testing arena (C57 WT) saline vs. adenosine: 100.00 ± 6.12% vs. 115.58 ± 10.05%, p = 0.231; (Casp-1 KO) saline vs. adenosine: 100.00 ± 9.60% vs. 115.02 ± 6.33%, p = 0.210; (IL-1R1 KO) saline vs. adenosine: 100.00 ± 12.91% vs. 108.02 ± 4.40%, p = 0.632; (BALB/c WT) saline vs. adenosine: 100.00 ± 12.43% vs. 107.98 ± 12.84%, p = 0.671; (A2A AR KO) saline vs. adenosine: 100.00 ± 9.26% vs. 97.20 ± 7.43%, p = 0.821. Fig.3.6C shows that mice administered adenosine 30 min prior to open field testing exhibited a 55% decrease in percentage of time spent in the open arms of the elevated zero-maze while Casp-1 KO, IL-1R1 KO and A2A AR KO mice were resistant (C57 WT) saline vs. adenosine: 100.00 ± 21.23% vs. 45.25 ± 13.74%, p = 0.05; (Casp-1 KO) 100.00 ± 15.30% vs. 101.47 ± 15.05%, p = 0.947; (IL-1R1 KO) saline vs. adenosine: 100.00 ± 22.91% vs. 139.16 ± 27.19%, p = 0.296; (BALB/c WT) saline vs.
adenosine: 100.00 ± 18.5% vs. 41.69 ± 7.82%, p = 0.027; (A2A AR KO) saline vs. adenosine: 100.00 ± 45.18% vs. 92.99 ± 14.44%, p = 0.887. Fig.3.6D demonstrates that adenosine did not curtail mouse locomotion within the elevated zero-maze (C57 WT) saline vs. adenosine: 100.00 ± 4.45% vs. 106.65 ± 5.52%, p = 0.371; (Casp-1 KO) saline vs. adenosine: 100.00 ± 2.89% vs. 119.70 ± 10.68%, p = 0.222; (IL-1R1 KO) saline vs. adenosine: 100.00 ± 3.08% vs. 103.72 ± 4.76%, p = 0.818; (BALB/c WT) saline vs. adenosine: 100.00 ± 17.37% vs. 108.53 ± 8.45%, p = 0.674; (A2A AR KO) saline vs. adenosine: 100.00 ± 5.18% vs. 113.77 ± 11.38%, p = 0.313.

**Discussion**

The novel data that we present show for the first time that the increase in anxiety (thigmotaxis), decrease in food intake and decrease in locomotor activity caused by adenosine are absent in mice with defects in the A2A AR, caspase-1, IL-1ß to IL-1R1 pathway. These data strongly implicate the induction of IL-1ß protein maturation as a causal mechanism for adenosine-linked behaviors. Our *ex vivo* data indicate that adenosine initiates the behavioral cascade by engaging the A2A AR, increasing intracellular cAMP, activating PKA and triggering K⁺ efflux. This signaling pathway is the initiating event for caspase-1 activation, IL-1ß protein elevation and subsequent behavioral changes, including anxiety.

Anxiety disorders and pro-inflammatory Th1-like responses have long been linked (Maes et al., 1998), but the pathways involved have remained elusive. Recently, adenosine receptors have been postulated to have a role in anxiety due to a variety of anecdotal accounts tying consumption of caffeinated drinks to self-perceived anxious/jittery behaviors (Braun et al., 2011, Lara et al., 2010, Maximino et al., 2011, Lee et al., 1985). However, as a competitive pan-AR
blocker, caffeine appears to afford protection against some neuroinflammatory/neurodegenerative diseases like Alzheimer’s disease (AD) (Arendash et al., 2006, Arendash & Cao, 2010, Eskelinen & Kivipelto, 2010) indicating that adenosine in the brain can act as a proinflammatory agent (Chiu et al., 2012, Yu et al., 2004). Since IL-1 is a well described anxiogenic (Connor et al., 1998, Koo & Duman, 2009), the proinflammatory actions of adenosine might, in fact, promote an anxious phenotype. Support for this contention is seen in Fig.3.1 where we show that perfused adenosine doubles brain caspase-1 activity confirming our recent finding that adenosine is an activator of caspase-1 in the brain (Chiu et al., 2012).

As part of the multiprotein inflammasomes, caspase-1 is a critical generator of mature IL-1β (Bauernfeind et al., 2009). How danger signals are recognized by the inflammasome is not entirely known but K⁺ efflux is an important trigger in caspase-1 activation (Gross et al., 2011). Fig.3.1 demonstrates that an excess of extracellular potassium prevents adenosine-dependent activation of caspase-1 as does the use of the K⁺ channel blocker glyburide (Lamkanfi et al., 2009). Thus, adenosine-dependent activation of caspase-1 appears dependent on K⁺ channel mediated potassium efflux in a manner similar to lipopolysaccharide (LPS)-treated macrophages where NLRP3 inflammasome-dependent activation of caspase-1 can be prevented by raising the extracellular K⁺ concentration above 70 mM (Pétrilli et al., 2007, Fernandes-Alnemri et al., 2007) or by antagonizing K⁺ channel with sulfonylureas (Lamkanfi et al., 2009, Lamkanfi & Dixit, 2009).

How LPS, which interacts with Toll-like receptor 4 (TLR4), causes K⁺ efflux is still unclear (Scheel et al., 2006, Jo et al., 2011), but for GPCRs, like the ARs, the mechanisms by
which K⁺ efflux is triggered is much better defined (Kleppisch & Nelson, 1995, Mathie, 2007). Importantly, the A2A AR is a well described inducer of K⁺ efflux through a pathway reliant on increased intracellular cAMP, activation of PKA and opening of K_{ATP} channels (Kleppisch & Nelson, 1995). Fig.3.1&2 show that the competitive PKA inhibitor KT5720 prevented adenosine-dependent-activation of caspase 1 and that caspase-1 activation was reliant on the A2A AR. These findings were not unexpected in light of our previous work showing that an A1/A2A AR inhibitor cocktail blocked adenosine-dependent activation of caspase-1 (Chiu et al., 2012). Since the A1 AR is a G_i-linked GPCR that inhibits intracellular cAMP production (Stiles, 1986), it was unlikely that the A1 AR was the upstream receptor responsible for adenosine-dependent caspase-1 activation in the brain. In general, ligand bound A2A ARs increase intracellular cAMP concentration (Wu et al., 2013). Therefore, adenosine-dependent activation of caspase-1 in the brain leading to increased brain IL-1β (Table 1) appears reliant on A2A ARs, PKA and K_{ATP} channel mediated K⁺ flux.

Central IL-1, especially in rodents, promotes anxiety-like behavior as first demonstrated in rats administered ICV IL-1β (Connor et al., 1998). Most studies examining the behavioral effects of IL-1 have been developed from the sickness behavior paradigm in which peripheral infection, often modelled by IP LPS administration, triggers brain-based production of IL-1. Fig.3.4 shows that IP administration of adenosine increases brain caspase-1 activity which was coupled to a 2-fold increase in serum adenosine. Importantly, these increases in adenosine and caspase-1 were linked to the classic IL-1-associated sickness symptoms of a transient decrease in locomotion and food intake that resolved by 75 mins post injection. Evidence that caspase-1 and IL-1 were responsible for these adenosine-dependent behaviors was gleaned from caspase-1 KO
and IL-1R1 KO mice which demonstrated a resistance to these adenosine-induced behaviors (Fig.3.4). Interestingly, caspase-1 KO mice appeared to have an inverse phenotype because food intake increased in response to adenosine. Since IL-1R1 KO mice did not show this same behavior, it is likely that another caspase-1-activated bioactive is involved. A probable candidate is IL-18 since pro-IL-18 is matured by caspase-1 (Sansonetti et al., 2000) and IL-18 KO mice have an obese phenotype (Netea et al., 2006). As was anticipated, A2A AR KO mice were resistant to adenosine-induced reductions in locomotion and food intake supporting our contention that adenosine through A2A AR activates caspase-1 leading to IL-1-dependent biobehaviors.

Fig. 3.5 shows that central administration of the caspase-1 inhibitor Ac-YVAD-CMK prevented adenosine-induced loss of locomotion and food intake. These findings support the role of brain-based caspase-1 and by extension IL-1β as the drivers of biobehaviors triggered by peripheral elevations in extracellular adenosine. Additional support for the above is derived from our previous work in which hypoxia/reoxygenation-induced extracellular adenosine caused adenosine-dependent activation of caspase-1 in the brain but not in the liver (Chiu et al., 2012). Since oxidative stress and other metabolic-related dysfunctions are the most common precipitates of elevated serum adenosine (Rego et al., 2000, Lavie, 2003, Barsotti & Ipata, 2004), our finding indicate that conditions unrelated to microbial driven inflammation can activate caspase-1 in the brain and trigger IL-1 associated adverse biobehaviors. Furthermore, while certain psychiatric conditions appear to emerge in association with proinflammatory conditions like obesity and type 2 diabetes (Anderson et al., 2001, Simon et al., 2006, Das-
Munshi et al., 2007, Petry et al., 2008), causation related to TLRs has not been clearly demonstrated suggesting that danger signals recognized by non-TLRs are important.

While induced reductions in spontaneous locomotion and ad libitum food intake are often associated with sickness symptoms (Dantzer et al., 2008), these relatively non-specific behaviors are seen in a variety of conditions unrelated to sickness including anxious-like states (Schneider et al., 2012). As Fig.3.6 demonstrates, adenosine decreased the time spend in the center area of the open field test but given the novel/stressful open field environment mouse locomotion in this test was not reduced. Similarly, adenosine-induced a decrease the time spent in the open arms of the elevated zero-maze with no effects on overall motor activity in the novel arena. These findings underscore the difference between adenosine-induced biobehaviors and those seen in classic TLR-mediated sickness symptoms or sickness behaviors secondary to IP IL-1β administration. Classic sickness usually does not manifest until 2 hrs post LPS or IL-1β administration and loss of function such as reduced locomotion cannot be normalized by changing the environment or provocation with a noxious stimulus (Dantzer et al., 2008). The cause for this response delay is that LPS and IL-1β do not cross the blood brain barrier efficiently and/or act peripherally to stimulate central IL-1β transcription prior to inflammasome activation (Layé et al., 1995). In contrast, peripheral adenosine can easily and rapidly access the CNS (Li et al., 2001) where without increasing IL-1 transcription (data not shown) it can, within 30 min, trigger anxiety like behavior in an A2A AR-, caspase-1- and IL-1R1-dependent fashion (Fig.3.6).
Although a direct role for adenosine in anxiety has not been previously demonstrated, such an action is suggested. In animal models, caffeine at a moderate dose (30 mg/kg) increases the time rats spend in the open arms of an elevated plus maze (Garcia et al., 2011) indicating that a blockade of adenosine signaling might be anxiolytic. Furthermore, zebra fish with early life anxiety-like behavior can be made less anxious with caffeine (Khor et al., 2013). However, why caffeine is controversial in the context of human anxiety may be due to the dose of caffeine and the natural distribution of adenosine receptors. For instance, zebra fish develop anxiety-like behaviors at a high dose of caffeine (100 mg/kg) but not at a low dose (10 mg/kg) (Maximino et al., 2011). Interestingly, administration of an A1 AR but not A2A AR antagonist increases anxiety-like behavior (Maximino et al., 2011), suggesting that unopposed activation of PKA is anxiogenic (Keil et al., 2012). Therefore, the anxiogenic effects of high-dose caffeine may be mediated via A1 inhibition which likely bypasses caffeine-mediated blockade of A2A AR signaling. In addition, as we show in Table 2 and others have demonstrated (Rosin et al., 1998), the location of A2A ARs in the brain is primarily in the amygdala and striatum and manipulation of amygdala-based neuronal activity can cause anxiety like behavior in mice (Tye et al., 2011). Finally, human anxiety has a link to adenosine. Coincident with elevated plasma adenosine is a higher prevalence of psychiatric mood disorders especially anxiety (Henningsen et al., 2003, Cella et al., 2011, Duley et al., 2000). In sum, physiological levels of extracellular adenosine are important to selectively inhibiting neurotransmission via hyperpolarization of excitatory synapses (Lambert & Teyler, 1991, Prince & Stevens, 1992). However, in deleterious conditions where excessive adenosine is present such as undue alcohol ingestion, sterile inflammation, tissue injury and hypoxia/ischemia (Winn et al., 1981, Karmouty-Quintana et al., 2013, Spinetta
et al., 2008) this same mechanism of hyperpolarization appears to trigger casapse-1 activation and generation of IL-1β that causes adverse biobehaviors that include anxiety.
CHAPTER 4: HYPOXIA/REOXYGENATION INCREASE CASPASE-1 ACTIVITY IN NEURONS THROUGH THE A2A ADENOSINE RECEPTOR CONTRIBUTING TO ANXIETY

Abstract

Anxiety is one of the most prevalent comorbidity associated with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Activation of the innate immune system is a known inducer of anxiety-like behaviors. In adult male mice, we found that hypoxia increased circulating adenosine concentration by more than 2.5-fold. Moreover, we see that hypoxic mice displayed four times greater staining for caspase-1 activity in the amygdala when compared to normoxic controls. Finally, hypoxia increased anxiety-like behaviors in wild-type mice by 45 and 86% as measured in the open field test and elevated zero-maze, respectively, while A2A AR KO mice were resistant to hypoxia-induced anxiety-like behaviors. Our results indicate that hypoxia induces anxiety-like behaviors by increasing adenosine signaling via A2A AR to increase caspase-1 activity.

Introduction

At a rate of 20 - 40%, anxiety disorders are more prevalent in patients of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) than the general public (Hough & Herridge, 2012; Kessler et al., 2005) where psychiatric symptoms are seen even 2 years after ARDS (Hopkins et al., 2010). Common in ARDS patients is the progression of inflammation as well as elevation in extracellular adenosine (Hart et al., 2008). Recently, we demonstrated that
increases in extracellular adenosine can trigger anxiety-like behaviors via caspase-1 activation and subsequent IL-1β release.

The loss of oxygen to the brain can activate the interleukin (IL)-1 arm of the neuroimmune system where psychological dysfunction such as amnesia and social withdrawal can occur (Johnson et al., 2007; Chiu et al., 2012). A common process involved in neuroimmune activation is the generation of the proinflammatory cytokine, IL-1β (Buttke & Sandstrom, 1994; Donath et al., 2008). Basal levels of brain-based IL-1 is required for cognitive function (Dantzer et al., 2008), while excessive concentration can be indicative of brain injury (Ma et al., 2003). IL-1 is involved in hypoxia and ischemia (Touzani et al., 1999), however, the mechanism involved in IL-1 release remains unclear. Previously, we have demonstrated that hypoxia and reoxygenation can lead to an increase in caspase-1 activity while this increase is dependent on activation of the adenosine receptors (Chiu et al., 2012). Caspase-1 exists as a proenzyme (Damiano et al, 2004) and requires proteolytical cleavage by the Nod-like receptor inflammasome (Miao et al, 2011). Once activated, active caspase-1 can produce the secretable form of IL-1β from pro-IL-1β (Bauernfeind et al, 2009).

Reoxygenation after hypoxia is can bolster recovery as well as inflict damage (González-Correa et al., 2007), cell death (Saikumar et al., 1998) and inflammation (Johnson et al., 2007, Chiu et al., 2012). Importantly, hypoxia/reoxygenation can increase extracellular adenosine from 30-300 nM to 10-50 μM concentration (Rudolphi et al., 1997; Hagberg et al., 1987; Guinzberg et al., 2006). Similarly, inflammation due to septic shock can also increase extracellular adenosine
as well as IL-1 (Martin et al., 2000; Cannon et al., 1990). Importantly, we previously showed that adenosine administration can elicit an increase in caspase-1 activity via A2A AR. Therefore, we examined the mechanism by which hypoxia/reoxygenation-induced anxiety-like behavior is reliant on AR and caspase-1.

Methods

Materials- All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) except as noted.

Animals- Animal use was conducted in accordance with Institutional Animal Care and Use Committee approved protocols at the University of Illinois. C57BL/6J (C57 WT) and BALB/cJ (BALB/c WT), A2A AR KO mice on a BALB/cJ background were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were group housed (×8 cage) in standard shoebox cages (length 46.9 cm; width 25.4 cm; height 12.5 cm) and allowed water and food ad libitum. Housing temperature (72 °F) and humidity (45–55%) were controlled, as was a 12/12 h reversed dark-light cycle (2200–1000 h). Video recording of animal behavior was performed under red light using a Sony HDR-XR500V Night Shot capable video camera (Tokyo, Japan). All treatments at all time points represent separate cohorts of mice. Mice were between 8 and 14 weeks of age and the total number of mice utilized was 125.
Hypoxia/Reoxygenation- As we have previously described (Sherry et al., 2009a; Sherry et al., 2009b; Chiu et al., 2012), mice (n = 16/episode) were transferred from their home cages to a BioSperix ProOx/A-Chamber Biological Atmosphere System (Biospherix, Lacona NY) and subjected to either a 6% oxygen + 94% nitrogen environment (hypoxia) or an atmospheric air environment (normoxia) for 2 h. After exposure mice were returned to their home cages.

Injectables- Biotin-YVAD-CMK (AnaSpec, CA) was administered ICV, as we have described (Johnson et al., 2007; Chiu et al., 2012), at a dose of 50 ng/μL/mouse, 30 min prior to hypoxia.

ICV cannulation- As we have described (Johnson et al., 2007; Chiu et al., 2012), mice were anesthetized with an IP injection of sodium ketamine hydrochloride/xylazine hydrochloride solution delivering 80 mg/kg ketamine and 12 mg/kg xylazine. Animals were placed in a David Kopf Instruments stereotaxic instrument (Tujunga, CA), Plastics One mouse-specific brain infusion cannulas (Roanoke, VA) were placed 0.6 mm posterior, 1.5 mm lateral to the bregma and to extend 2.5 mm ventral from the surface of the skull. Cannulas were fixed to the skull with Plastics One cyanoacrylate gel adhesive and protected by a Plastics One guard. Mice were allowed 7 days to recover prior to treatment.

Serum adenosine – As we have previously described. In brief, 500 μL of blood was mixed 1:2 with 138 mM NaCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, 3 mM KCl, 5.9 mM NaHCO₃, 5.6 mM dextrose, 2.15 mM CaCl₂, 1% DMSO, and 120 μM dipyridamole. Samples were centrifuged at 3650 x g for 10 min at 4°C and proteins precipitated by addition of 2% v/v of 70%
(12N) perchloric acid with re-centrifuged at 3650 x g for 10 min at 4°C. Adenosine was
determined on a 5500 QTRAP LC/MS/MS (AB Sciex, Foster City, CA) with a 1200 series
HPLC (Agilent Technologies, Santa Clara, CA). LC separation was performed on a Phenomenex
Kinetex 2.6 u PFP column (4.6 x 100mm) (Torrence, CA). Mobile phase A was 0.1% formic
acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. Flow rate was 0.3 mL/min
and the linear gradient was: 0-1 min, 100% A; 5 min, 90% A; 10 min, 80% A; 12-18 min, 0% A;
and 18.5-25 min, 100% A with an auto-sampler temperature of 5°C. The injection volume was 5
μL. Mass spectra were acquired with positive electrospray ionization with an ion spray voltage
of 5500 V at a source temperature of 450 °C. The curtain gas, ion source gas 1 and ion source gas
gas 2 were 35, 50, and 65, respectively. Multiple reaction monitoring was used to quantify adenosine
m/z 268.1 --> m/z 136.1, inosine m/z 269.1 --> m/z 137.1 and internal standard caffeine m/z
195.1 --> m/z 138.1.

Activated caspase-1 labeling- Similar to methods we have previously described (Johnson
et al, 2007; Chiu et al., 2012). 7 days after surgery, mice were injected with 50ng of Biotin-
YVAD-CMK ICV 30 min prior to hypoxia. After 2 hours of 6% oxygen, mice were taken out of
the hypoxia chambers and allowed to reoxygenate for 4 hours. After reoxygenation, mice were
euthanized using CO₂ and perfused with 4% paraformaldehyde. Brains were removed from 0.7
to -2.0 mm from the bregma coronally using a Mouse Brain Slice (Zivic Instruments, Pittsburgh,
PA). Slices were fixed in 4% paraformaldehyde for 24 hours then paraffin embedded. 5μm
sections at 0.5 and -1.8 mm from the bregma were treated with antigen retrieval (BioGenex
Antigen Retrieval Citra Plus) and blocked with 3% hydrogen peroxide in methanol and labeled
with SS HRP Label (peroxidase-conjugated Streptavidin HK330-9KT), stained with liquid 3.3’-
diaminobenzidine (DAB) chromogen (HK124-7K), counterstained with hematoxylin and mounted and coverslipped with the BioGenex i6000 Automated Staining system (BioGenex, San Ramon, CA). The entire slide was imaged at 40x with a NanoZoomer 2.0-HT (Hamamatsu, Bridgewater, NJ). Area and intensity of staining was measured using ImageJ (National Institute of Health).

*Open field test*- Open field testing was performed as we have described (York et al., 2012). In brief, mice were placed in a novel open field arena (66 cm length x 45.7 cm width x 22.9 cm height) 30 min after adenosine injection. Lighting was positioned to create a 9 cm shadow from each side wall. Mice were video recorded for 5 min. Total distance traveled, time spent in the non-shadowed and shadowed areas of the arena were determined using Noldus Information Technology EthoVision XT 7. Results are presented as percent control of time (sec) in the non-shadowed area (center).

*Elevated zero-maze*- Elevated zero-maze testing was performed as we have described (York et al., 2012). In brief, 30 min after adenosine injection mice were placed in a circular elevated zero-maze (22.5 cm in diameter, track width of 2.75 cm, elevated 33 cm above the floor). The maze was divided into two open quadrants and two enclosed quadrants. Mice were video recorded for 5 min. Total distance traveled, time spent in the open and closed arms of the maze were determined using Noldus Information Technology EthoVision XT 7. Results are presented as percent control of time (sec) in the open arms.
Statistics- All data are presented as mean ± SEM. Data was analyzed using Sigma Plot 11.2 (Systat Software, Chicago, IL). To test for statistical differences, a one-way or two-way ANOVA was with repeated measurements where needed. Tukey’s test was used for post-hoc pair-wise multiple comparison procedures. Where indicated, raw data was transformed to attain equal variance. Statistical analysis included testing for time x treatment interactions when needed. Statistical significance was denoted at p < 0.05.

Results

Hypoxia/reoxygenation-induced elevation in circulating adenosine- Fig.4.1 demonstrates that circulating adenosine is elevated by 264% 30 min after hypoxia and returned to basal levels by 2 hours. Main effect of hypoxia, p = 0.049, main effect of time, p = 0.031 main effect of interaction, p = 0.087 (normoxic vs. hypoxic at 0.5 hr, 0.45 ± 0.09 vs. 1.18 ± 0.28, p = 0.005; normoxic vs. hypoxic at 2 hr, 0.35 ± 0.14 vs. 0.40 ± 0.04, p = 0.854; normoxic 0.5 hr vs. normoxic 2 hr, 0.45 ± 0.09 vs. 0.35 ± 0.14, p = 0.705; hypoxic 0.5 hr vs. hypoxic 2 hr, 1.18 ± 0.28 vs. 0.40 ± 0.04, p = 0.010).

Hypoxia/reoxygenation-induced activation of caspase-1 in neurons- Fig.4.2A&B are representative slides from the amygdala of normoxic and hypoxic mice when stained for active caspase-1. Fig 2C shows that the amygdala from hypoxic mice displayed a 4-fold increase in the area stained for active caspase-1 when compared to normoxic control (normoxic vs. hypoxia, 2640.46 ± 300.42 vs. 11184.18 ± 872.36, p < 0.001). Fig.4.2D shows that the average staining intensity is 111% higher in hypoxic mice when compared to normoxic control (normoxic vs. hypoxic, 173.74 ± 3.97 vs. 193.01 ± 4.63, p = 0.030).
Hypoxia/reoxygenation-induced anxiety is mediated via A2A AR - Fig. 4.3A shows that mice exposed to hypoxia and allowed to reoxygenate for 2 hours displayed a 45% decrease in time spent in the center arena when compared to normoxic controls (normoxic WT vs. hypoxic WT, 100.00 ± 13.95 vs. 56.34 ± 12.45, p = 0.048), while A2A AR KO mice are protected against anxiety-like behavior (normoxic A2A AR KO vs. hypoxic A2A AR KO, 100.00 ± 28.25 vs. 104.23 ± 19.84, p = 0.905). WT mice allowed to reoxygenate for 4 hours were not significantly different from normoxic controls (normoxic WT vs. hypoxic WT, 100.00 ± 12.03 vs. 69.90 ± 17.92, p = 0.200; normoxic A2A AR KO vs. hypoxic A2A AR KO, 100.00 ± 21.74 vs. 92.05 ± 19.25, p = 0.793). Fig. 4.3B shows that mice exposed to hypoxia and allowed to reoxygenate for 2 hours displayed a 86% decrease in time spent in the open arms of the elevated zero maze when compared to normoxic controls (normoxic WT vs. hypoxic WT, 100.00 ± 24.01 vs. 13.95 ± 9.45, p = 0.033), while A2A AR KO mice are protected against anxiety-like behavior (normoxic A2A AR KO vs. hypoxic A2A AR KO, 100.00 ± 34.63 vs. 161.64 ± 18.21, p = 0.166). WT mice allowed to reoxygenate for 4 hours were not significantly different from normoxic controls (normoxic WT vs. hypoxic WT, 100.00 ± 11.83 vs. 105.55 ± 28.17, p = 0.798; normoxic A2A AR KO vs. hypoxic A2A AR KO, 100.00 ± 14.46 vs. 217.88 ± 43.47, p = 0.038).

Discussion

For the first time, we show, visually, that hypoxia/reoxygenation increases caspase-1 activity in the neurons in the amygdala. Importantly, hypoxia/reoxygenation increased circulating adenosine and induces anxiety-like behaviors in WT but not A2A AR KO mice. These data strongly suggests a causative mechanism of hypoxia/reoxygenation-induced
adenosine release and subsequent increase in caspase-1 activity to produce IL-1β to elicit anxiogenic effects.

ARDS is a severe reaction to lung injury or infection. It can cause inflammation due to decreased oxygen that has a 90% death rate when left untreated where survival increase to 50-75% with treatment (Guérin et al., 2013). Extracellular adenosine concentration increases in ARDS due to increased degradation of purine nucleotides (Guckelberger et al., 2004; Hart et al., 2008) where at low levels can serve as a neuroprotectant. Similar to the study by Hagberg et al. (Hagberg et al., 1987), Fig.4.1 shows that circulating adenosine is increased after hypoxia, although not to the same degree. This discrepancy may be due to the fact that Hagberg used complete brain ischemia for 15 min, where oxygen supply is cut off, and sampled via microdialysis from the brain, while this study measured circulating adenosine after 6% hypoxia. Our less severe treatment may have caused a lower increase in adenosine. Our normoxic controls showed concentrations near the range published by Rudolph et al. (Rudolphi et al., 1992), proving validity to our collection method.

As we have previously published, hypoxia/reoxygenation leads to a region-specific increase in caspase-1 activity (Chiu et al., 2012). This was previously measured colorimetrically for changes in activity in vitro. Here, we present a novel method of detecting caspase-1 in the brain. Biotin-YVAD-CMK is a caspase-1 specific inhibitor that covalently and irreversibly binds to mature but not pro-caspase-1 (Gao et al., 2008l Wu et al., 2010). We see that labeling biotin with strepavidin-HRP and staining with DAB allows us to visualize active caspase-1 in vivo.
When looking at various regions of the brain, we see that the amygdala (Fig.4.2), and parts of the striatum, but not the hippocampus (data not shown), showed increase caspase-1 staining due to hypoxia. This confirms our finding from 2012 that hypoxia/reoxygenation activates caspase-1 in specific regions rather than the whole brain.

Interestingly, we see that caspase-1 activity is stained most visibly in neurons. Previously, we saw that inhibition of A2A AR signaling can be protective against hypoxia/reoxygenation- (Chiu et al., 2012) and adenosine-induced caspase-1 activation, and here, we see that A2A AR KO mice are protected against hypoxia/reoxygenation-induced anxiety-like behavior, we expected to see more ubiquitous activation due to the presence of A2A AR in microglia (Orr et al., 2009), astrocytes (Brambilla et al., 2003), endothelial cells (Mills et al., 2011), and oligodendrocytes (Melani et al., 2009). While we expected to see staining in immune cells due to caspase-1 and IL-1β regulation, this is not too surprising because, as we have previously shown, 6% hypoxia/reoxygenation does not elicit gliosis (Chiu et al., 2012) or cell death (Johnson et al., 2007).

As we see in Fig.4.2, the amygdala of hypoxic mice displayed increased active caspase-1 staining. This suggests an increase in IL-1β production, where in the brain can be anxiogenic (Song et al., 2003). In humans, anxiety and mood disorders also coincide with elevated plasma adenosine (Cella et al., 2011, Duley et al., 2000). Importantly, we and others (Rosin et al., 1998) have shown that the A2A AR is primarily expressed in the amygdala and striatum. In fact, Tye et al. demonstrated that neurons in the amygdala regulate anxiety-like behaviors (Tye et al., 2011).
This is shown in Fig. 4.3, where hypoxia/reoxygenation induced anxiety-like behaviors in the open field test as well as the elevated zero maze. Furthermore, A2A AR KO mice were protected against this anxiogenic effect, further suggesting the importance of the A2A AR in regulating hypoxia/reoxygenation-induced behavioral changes.

In conclusion, pathological states such as sterile infection and hypoxia/ischemia can not only induce inflammatory responses but also increase in extracellular adenosine (Winn et al., 1981, Karmouty-Quintana et al., 2013) to hyperpolarize and inhibit neuronal activity (Lambert & Teyler, 1991, Prince & Stevens, 1992). The data presented here demonstrates that hypoxia/reoxygenation induces an elevation in circulating adenosine that can act upon the neurons in the amygdala to increase caspase-1 activity and subsequently generate IL-1β-induced anxiety-like behaviors.
Fig. 1.1. Purine metabolism and adenosine signaling pathway.
Table 1.1. Effects of adenosine in on neurological disorders

<table>
<thead>
<tr>
<th>Disease</th>
<th>Experimental Model</th>
<th>Interaction with Adenosine</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Type 1 Diabetes</td>
<td>Nonobese diabetic rats (streptozotocin-induced hyperglycemia) hippocampal slice culture</td>
<td>Diabetic-like state enhanced adenosine-evoked inhibition</td>
<td>Morrison et al., 1992</td>
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<td>Alzheimer's Disease</td>
<td>Alzheimer's Disease Patients</td>
<td>AD patients showed increased expression of A1 and A2A receptors. A1 agonist increased production of soluble amyloid precursor protein</td>
<td>Angulo et al., 2003</td>
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<td></td>
<td>Cultured Neurons</td>
<td>Caffeine protects against β-amyloid-induced toxicity. A2A but not A1 antagonism protected against β-amyloid-induced toxicity</td>
<td>Dall'Igna et al., 2003</td>
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<tr>
<td></td>
<td>ICV administration of β-amyloid (Mouse)</td>
<td>12-d caffeine treatment in drinking water plus acute caffeine injection prevented β-amyloid-induced cognitive impairment. 4-d caffeine of A2A antagonist injection prevented β-amyloid-induced cognitive impairment</td>
<td>Dall'Igna et al., 2007</td>
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<td></td>
<td>Striatum from AD patients</td>
<td>Loss of A1 receptors in AD striatum paralleled a loss of choline acetyltransferase activity</td>
<td>Ikeda et al., 1993</td>
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<td>APPsw transgenic mice</td>
<td>Daily caffeine intake improved behavior and reduced hippocampal β-amyloid</td>
<td>Arendash et al., 2006</td>
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<tr>
<td>Disease</td>
<td>Experimental Model</td>
<td>Interaction with Adenosine</td>
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<td>Parkinson's Disease</td>
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<td>Heteromeric antagonistic D2/A2A receptors</td>
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<td>A2A opposes the action of D2 on GABAergic neurons</td>
<td>Schwarzschild et al., 2006</td>
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<td>Heteromeric synergistic A2A/mGlu receptors</td>
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<td>Slow down progression of PD</td>
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<td>KW-6002 (A2A antagonist) shows potential in phase IIb and III in PD patients</td>
<td>Jacobson &amp; Gao, 2006</td>
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<td>Depression</td>
<td>Various</td>
<td>Adenosine antagonism delayed hypoxia-induced depression</td>
<td>Fowler, 1989</td>
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<td></td>
<td>Adenosine antagonism exhibits antidepressant-like properties</td>
<td>Sarges et al., 1990</td>
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<td>A2A KO displayed less depressive-like behavior when compared to wild-type control</td>
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<td>D2 antagonist prevented A2A antagonist's antidepressive-like properties</td>
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<td></td>
<td></td>
<td>Adenosine antagonism delayed hypoxia-induced depression</td>
<td>Fowler, 1989</td>
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Fig. 2.1. *Restoration of memory recall after acute hypoxia.* (A) Wild type mice were trained in memory formation using novel object recognition 1 h prior to hypoxia. Mice were then exposed to normoxia or hypoxia for 2 hrs. Memory recall (percent investigation) was measured at the reoxygenation time points indicated. Results are expressed as means ± s.e.m.; n = 8. Bars without a common superscript are different (p < 0.05). (B) Mice were treated as in A and spontaneous locomotor activity (total distance traveled) was measured at the reoxygenation time points indicated. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05).
Fig. 2.2. Memory formation recovers more slowly than memory recall after acute hypoxia.

(A) Wild type mice were exposed to normoxia (Norm) or hypoxia for 2 hrs. After hypoxia, mice were trained in memory formation using novel object recognition 1 h prior to the time points indicated. Memory recall (percent investigation) was measured at the reoxygenation time points indicated. Results are expressed as means ± s.e.m.; n = 6-8. Bars without a common superscript are different (p < 0.05).

(B) Mice were treated as in A. Mice were trained in memory formation using cued fear conditioning after 4 hrs of reoxygenation. Memory recall (immobility) was measured after 5 and 52 h of reoxygenation. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (P < 0.05).

(C) Mice were treated as in A. Perfect alternations were measured after 4, 52, and 76 h of reoxygenation. Results are expressed as means ± s.e.m.; n = 4.
Fig. 2.3. Knockout of IL-1R1 improves memory formation and locomotion while blunting activation of ERK1/2 and p38 MAPK. (A) Wild type (WT) or IL-1R1 KO mice were exposed to normoxia or hypoxia for 2 hrs. Mice were trained in memory formation using novel object recognition after 4 hrs of reoxygenation. Memory recall (percent investigation) was measured after 5 hrs of reoxygenation. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) WT mice treated with/without IP IL-1RA were
exposed to normoxia or hypoxia as in A and memory was tested as in A. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05). (C) WT mice treated with/without ICV Ac-YVAD-CMK were exposed to normoxia or hypoxia as in A and memory was tested as in A. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05). (D) WT or IL-1R1 KO mice were treated as in A and spontaneous locomotor activity (total distance traveled) was measured at the reoxygenation time points indicated. Results are expressed as means ± s.e.m.; n =4. Bars without a common superscript are different (p < 0.05). (E) WT and IL-1R1 KO mice were exposed to normoxia or hypoxia as in A. Brain p-pERK1/2, p-p38 MAPK and p-JNK were measured 1 hr after hypoxia. Results are expressed as means ± s.e.m.; n = 6-9. Bars without a common superscript are different (p < 0.05).
Fig. 2.4

A

Reoxygenation Time (h)

Caspase 1 Activity (Relative)

0.0
0.5
1.0
1.5
2.0
2.5

Normoxic
Hypoxic

B

Saline Normoxic
Saline Hypoxic
Caffeine Normoxic
Caffeine Hypoxic

Caspase 1 Activity (Relative)

0.0
0.5
1.0
1.5
2.0
2.5
3.0

a
b

C:

Vehicle Normoxic
Vehicle Hypoxic
A1/A2A antag Normoxic
A1/A2A antag Hypoxic

Caspase 1 Activity (Relative)

0.0
0.5
1.0
1.5
2.0
2.5
3.0

a
b

a
a
a
a
AR blockade prevents hypoxia-dependent activation of caspase 1 in the brain. (A) Wild type mice were exposed to normoxia or hypoxia for 2 hrs. Caspase 1 activity was measured at the reoxygenation time points indicated. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) Wild type mice treated with/without caffeine were exposed to normoxia or hypoxia as in A and caspase 1 activity measured 1 hr after reoxygenation. Results are expressed as means ± s.e.m.; n = 4. Bars without
a common superscript are different (p < 0.05). (C) Wild type mice treated with/without 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine (A1/A2A antag) were exposed to normoxia or hypoxia as in A and caspase 1 activity measured 1 hr after reoxygenation. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (D) Wild type mice treated with/without NAC were exposed to normoxia or hypoxia as in A and caspase 1 activity measured 1 hr after reoxygenation. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05). (E) Wild type mice were perfused with the adenosine concentrations indicated. Caspase 1 activity was measured 1 hr after perfusion. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05). (F) Wild type mice were perfused with/without 50 µM adenosine + 500 µM caffeine. Caspase 1 activity was measured 1 hr after perfusion. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05). (G) Wild type mice were perfused with/without 50 µM adenosine + 500 µM NAC. Caspase 1 activity was measured 1 hr after perfusion. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05).
Fig. 2.5. *Hypoxia induces brain region-specific activation of caspase 1* - (A) Wild type mice were exposed to normoxia or hypoxia for 2 hrs. Caspase 1 activity was measured in the amygdala and hippocampus 1 hr after reoxygenation. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) Wild type (WT) mice and IL-1R1 KO mice were treated as in A. Immunohistochemistry was performed for GFAP at 1 and 6 hrs after reoxygenation. Representative images of the amygdala (n = 3).
Fig. 2.6. AR blockade speeds recovery of memory formation after hypoxia. (A) Wild type mice treated with/without caffeine were exposed to normoxia or hypoxia for 2 hrs. Mice were trained in memory formation using novel object recognition after 4 hrs of reoxygenation. Memory recall (percent investigation) was measured after 5 hrs of reoxygenation. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05).

(B) Wild type mice treated with/without 8-cyclopentyl-1, 3-dipropylxanthine + 3, 7-dimethyl-1-propargylxanthine (A1/A2A antag) were exposed to normoxia or hypoxia as in A and memory tested as in A. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p < 0.05).
### Table 2.1 Impact of reoxygenation on the glutathione to glutathione disulfide (GSH/GSSG) ratio in the brain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normoxic</th>
<th>Reoxygenation (1 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.00 ± 0.21(^a)</td>
<td>0.49 ± 0.12(^b)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.88 ± 0.15(^a)</td>
<td>0.87 ± 0.10(^a)</td>
</tr>
<tr>
<td>NAC</td>
<td>0.82 ± 0.02(^a)</td>
<td>1.03 ± 0.03(^a)</td>
</tr>
</tbody>
</table>

Wild type mice were exposed to normoxia or hypoxia for 2 hrs. Whole brains were harvested and analyzed for GSH and GSSG concentrations. Results are expressed as relative change in GSH/GSSG ratio, means ± s.e.m.; n = 4. Results within individual rows without a common superscript are different (p < 0.05).
Fig. 3.1 *Inhibition of potassium efflux prevents adenosine-dependent activation of caspase-1 in the brain.* (A) Wild type mice were perfused with or without PBK and/or adenosine and activity of caspase-1 measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) Wild type mice were perfused with or without glyburide and/or adenosine and activity of caspase-1 measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05).
Fig. 3.2 The PKA inhibitor KT5720 blocks activation of caspase-1 by adenosine. (A) Wild type mice were perfused with or without KT5720 and/or adenosine and activity of caspase-1 measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) Wild type mice were perfused with or without adenosine and activity of PKA. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (C) Wild type mice were perfused with or without adenosine and the concentration of cAMP measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05).
Fig. 3.3 Activation of caspase-1 by adenosine is blocked by selective A2A AR antagonism and in A2A AR KO mice. (A) Wild type mice were perfused with or without 8-(3-Chlorostyryl)caffeine (CSC) and/or and activity of caspase-1 measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). (B) Wild type and A2A AR KO mice were perfused with or without adenosine and activity of caspase-1 measured. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05).
Adenosine activates caspase-1 in the brain while reducing locomotor activity and food intake. (A) Wild type mice were treated with or without IP adenosine and activity of caspase-1 in brain measured 15 min after adenosine administration. Results are expressed as means ± s.e.m.; n = 4. Bars without a common superscript are different (p < 0.05). Data was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks to attain equal variance. (B) C57 WT mice were treated with or without IP adenosine and locomotion measured at 15, 30, 45, 60, 75, 90 and 105 mins after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 8. *p < 0.05. (C) Casp-1 KO mice were treated with or without IP adenosine and locomotion measured at 15, 30, 45 and 60 mins after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 8. (D) IL-1R1 KO mice were treated with or without IP adenosine and locomotion measured at 15, 30, 45 and 60 mins after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 8. (E) BALB/c WT mice were treated with or without IP adenosine and locomotion measured at 15, 30, 45 and 60 mins after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 5. *p < 0.05. (F) A2A AR KO mice were treated with or without IP adenosine and locomotion measured at 15, 30,
45 and 60 mins after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 5. (G) C57 WT, Casp-1 KO and IL-1R1 KO mice were treated with or without IP adenosine and food intake measured for 60 min following adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls; n = 5-8. *p < 0.05. (H) BALB/c WT and A2A AR KO mice were treated with or without IP adenosine and food intake measured for 60 min following adenosine administration. Results are expressed as means ± s.e.m. versus saline injected controls l; n = 8. *p < 0.05.
Fig. 3.5 *Central administration of the caspase-1 inhibitor Ac-YVAD-CMK blocks adenosine-induced reductions in locomotor activity and food intake.* (A) C57 WT mice were treated with or without ICV Ac-YVAD-CMK and IP adenosine. Locomotion was measured at 0, 15, 30 and 45 mins after adenosine administration. Results are expressed as means ± s.e.m.; n = 5. Bars without a common superscript are different (p < 0.05). (B) C57 WT mice were treated with or without ICV Ac-YVAD-CMK and IP adenosine. Food intake was measured for 60 min after adenosine administration. Results are expressed as means ± s.e.m.; n = 5. Bars without a common superscript are different (p < 0.05).
Fig. 3.6 Adenosine causes anxiety-like behavior. (A) C57 WT, Casp-1 KO, IL-1R1 KO, BALB/c WT and A2A AR KO mice were treated with or without IP adenosine and time spent in the center area was measured 30 min after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected genotype control; n = 3-7. *p < 0.05. (B) C57 WT, Casp-1 KO, IL-1R1 KO, BALB/c WT and A2A AR KO mice were treated with or without IP adenosine. Total distance traveled in the arena was measured 30 min after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected genotype control; n = 3-7. (C) C57 WT, Casp-1 KO, IL-1R1 KO, BALB/c WT and A2A AR KO mice were treated with or without IP adenosine and time spent in the open arms of the elevated zero-maze were measured 30 min after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected...
genotype control; n = 4-6. *p < 0.05. Data was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks to attain equal variance. (D) C57 WT, Casp-1 KO, IL-1R1 KO, BALB/c WT and A2A AR KO mice were treated with or without IP adenosine. Total distance traveled in the elevated zero-maze was measured 30 min after adenosine administration. Results are expressed as means ± s.e.m. versus saline injected genotype control; n = 4-6.
Table 3.1 Adenosine increases IL-1β protein (pg/μg) in the brain but not the liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline (mean ± SEM)</th>
<th>Adenosine (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.71 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>2.00 ± 0.63</td>
<td>1.15 ± 0.29</td>
</tr>
</tbody>
</table>

Results are expressed as means; n = 6. Values without a common superscript are different; p < 0.05

Table 3.2 Differential expression of A2A AR mRNA

<table>
<thead>
<tr>
<th>Regions</th>
<th>Fold Change Mean (S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td>1.00 (0.12)&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.31 (0.38)&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.13 (0.03)&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means; n = 4-6. Values without a common superscript are different; p < 0.05
Fig.4.1. *Hypoxia/reoxygenation-induced elevation in circulating adenosine*. Wild type mice were exposed to normoxia (Normoxic) or hypoxia (Hypoxic) for 2 hrs. After hypoxia, mice were allowed to reoxygenate for 0.5 or 2 hrs as indicated before being euthanized and circulating adenosine concentration was measured. Results are expressed as means ± s.e.m.; n = 4-8. *p < 0.05.
Fig. 4.2. Hypoxia/reoxygenation-induced activation of caspase-1 in neurons. (A&B) Wild type mice were exposed to normoxia (Normoxic) or hypoxia (Hypoxic) for 2 hours. After
hypoxia, mice were allowed to reoxygenate for 4 hrs before euthanized and brain sections were taken. Representative images of the amygdala (n = 3-4). (C) Mice were treated as A. Immunohistochemistry was performed for Biotin-YVAD-CMK. Area of staining was measured using ImageJ. Results are expressed as means ± s.e.m.; n = 3-4. *p < 0.05. (D) Mice were treated as A. Immunohistochemistry was performed for Biotin-YVAD-CMK. Mena intensity of staining was measured using ImageJ. Results are expressed as means ± s.e.m.; n = 3-4. *p < 0.05.
Fig. 4.3. Hypoxia/reoxygenation-induced anxiety is mediated via A2A AR. (A) BALB/c WT and A2A AR KO mice were exposed to normoxia (Normoxic) or hypoxia (Hypoxic) and
(A) 

Time spent in the center area was measured after 2 and 4 hrs of reoxygenation as indicated. Results are expressed as means ± s.e.m. versus normoxic genotype control; n = 4-6. *p < 0.05.

(B) 

BALB/c WT and A2A AR KO mice were exposed to normoxia (Normoxic) or hypoxia (Hypoxic) and time spent in the open arms of the elevated-zero maze was measured after 2 and 4 hrs of reoxygenation as indicated. Results are expressed as means ± s.e.m. versus normoxic genotype control; n = 3-8. *p < 0.05.
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