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Research Report

Adenosine A1 receptor activation mediates NMDA receptor activity in a pertussis toxin-sensitive manner during normoxia but not anoxia in turtle cortical neurons

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ABSTRACT

Adenosine is a defensive metabolite that is critical to anoxic neuronal survival in the freshwater turtle. Channel arrest of the *N*-methyl-D-aspartate receptor (NMDAR) is a hallmark of the turtle's remarkable anoxia tolerance and adenosine A1 receptor (A₁R)-mediated depression of normoxic NMDAR activity is well documented. However, experiments examining the role of A₁Rs in regulating NMDAR activity during anoxia have yielded inconsistent results. The aim of this study was to examine the role of A₁Rs in the normoxic and anoxic regulation of turtle brain NMDAR activity. Whole-cell NMDAR currents were recorded for up to 2 h from turtle cortical pyramidal neurons exposed to pharmacological A₁R or G_i protein modulation during normoxia (95% O₂/5% CO₂) and anoxia (95% N₂/5% CO₂). NMDAR currents were unchanged during normoxia and decreased 51±4% following anoxic exposure. Normoxic agonism of A₁Rs with adenosine or N⁶-cyclopentyladenosine (CPA) decreased NMDAR currents 57±11% and 59±6%, respectively. The A₁R antagonist 8-cyclopentyl-1,3-dimethylxanthine (DPCPX) had no effect on normoxic NMDAR currents and prevented the adenosine and CPA-mediated decreases in NMDAR activity. DPCPX partially reduced the anoxic decrease at 20 but not 40 min of treatment. The G_i protein inhibitor pertussis toxin (PTX) prevented both the CPA and anoxia-mediated decreases in NMDAR currents and calcium chelation or blockade of mitochondrial ATP-sensitive K⁺ channels also prevented the CPA-mediated decreases. Our results suggest that the long-term anoxic decrease in NMDAR activity is activated by a PTX-sensitive mechanism that is independent of A₁R activity.

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Abbreviations: A₁R, Adenosine A1 receptor; ADO, Adenosine; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; [Ca²⁺]_i, Intracellular calcium concentration; DOR, Delta opioid receptor; ECD, Excitotoxic cell death; mKATP, Mitochondrial ATP-sensitive K⁺ channel; NMDAR, N-methyl-D-aspartate receptor; PTX, Pertussis toxin

1. Introduction

Adenosine has been termed a retaliatory metabolite because its concentration is rapidly elevated during periods of cell stress, which generally up-regulates cytoprotective mechanisms against anoxic insults and during seizures (Buck, 2004; Downey et al., 2007; Pagonopoulou et al., 2006). Freshwater turtles (*Chrysemys picta bellii* and *Trachemys scripta elegans*) are the most anoxia-tolerant vertebrates known and within 2 h of anoxic exposure brain [adenosine] increases 10-fold (Buck and Pamerter, 2006; Nilsson and Lutz, 1992). Furthermore, in contrast to the mammal brain, turtle brain adenosine receptor abundance is maintained while receptor binding affinity increases 2.5-fold during prolonged anoxic exposure (Lutz and Kabler, 1997; Lutz and Manuel, 1999). Increased [adenosine] and subsequent activation of the A₁R are critical to surviving anoxic insults in turtle cortex since blockade of these receptors during anoxia leads to increased K⁺ efflux, reactive oxygen species production, glutamate release and cell death (Milton et al., 2007; Pek and Lutz, 1997; Perez-Pinzon et al., 1993; Thompson et al., 2007). However, despite considerable evidence supporting a role for adenosine in the turtle's anoxia tolerance, the mechanisms underlying this protection are not well understood.

One potential mechanism of adenosine-mediated neuroprotection may be down-regulation of *N*-methyl-D-aspartate receptor (NMDAR) activity. In anoxia-sensitive mammals, decreased oxygen leads to elevated glutamate release and excitotoxic cell death (ECD). ECD is characterized by toxic accumulation of cytosolic Ca²⁺ ([Ca²⁺]_c) mediated by over-excitation of NMDARs (Choi, 1994, 1992). However, in turtle brain, toxic NMDAR-mediated Ca²⁺ accumulation is avoided due to a combination of maintained glutamate release and channel arrest of the NMDAR and the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA), activation of which removes the Mg²⁺ block from the NMDAR pore domain essential for NMDAR activation (Bickler et al., 2000; Milton et al., 2002; Pamerter et al., 2007, 2008a,b). While there is evidence supporting anoxic channel arrest of these two receptors, the mechanisms underlying this phenomenon have not been fully elucidated and may involve adenosine.

There is some evidence for adenosine involvement in the regulation of NMDAR activity in the turtle cortex. During normoxia, adenosine perfusion or activation of A₁Rs depresses NMDAR-mediated Ca²⁺ currents and reduces NMDAR channel open probability (P_{open}) by ~50%. Anoxia induces similar changes in NMDAR-mediated Ca²⁺ entry and NMDAR P_{open}, but concomitant perfusion of adenosine during anoxia does not induce synergistic increases in these two variables; suggesting the adenosine-mediated and the anoxia-mediated NMDAR regulatory mechanisms function via the same pathway (Bickler et al., 2000; Buck and Bickler, 1998, 1995). However, while the normoxic adenosine and A₁R agonist-mediated changes were blocked by an A₁R antagonist, the effects of A₁R blockade on the anoxic changes were inconsistent. A₁R antagonists prevented the anoxic decrease in NMDAR P_{open} but NMDAR-mediated Ca²⁺ currents remained depressed by anoxia. Thus while support for normoxic adenosine regulation of NMDAR activity is strong, data regarding the anoxic interaction between these receptors is inconclusive.

Other labs have also reported similarly dichotomous results regarding the role of adenosine in normoxia versus anoxia in facultative anaerobes. For example, in anoxic freshwater turtle brain, extracellular glutamate levels decrease due to reductions in synaptic vesicular glutamate release and reuptake (Milton et al., 2002; Nilsson et al., 1990). During normoxia, adenosine perfusion reduces synaptic glutamate release, however A₁R blockade does not prevent the anoxic effect (Milton et al., 2002). Another example comes from the hypoxia-tolerant coral reef epaulette shark, which can survive severe hypoxia for hours (Wise et al., 1998). Anoxia or adenosine induces significant and similar decreases in aortic blood pressure and heart rate; yet adenosine receptor blockade prevents only the normoxic adenosine-mediated changes (Stenslokken et al., 2004).

An explanation for these results may be derived from the mechanism of adenosine receptor activation. A₁Rs are G protein-coupled receptors and adenosine binding results in dissociation of an inhibitory G_i subunit, which interacts with adenylyl cyclase and depresses cAMP production. In addition to adenosine receptors there are other G protein-coupled receptors in turtle neurons that may converge on a common G_i protein-based pathway to regulate NMDAR activity during normoxia and anoxia. G_i proteins regulate cAMP expression, which in turn alters the activity of numerous downstream kinases and phosphatases (Domanska-Janik et al., 1993). In the anoxic turtle brain [cAMP] is decreased and the anoxic decrease in NMDAR activity involves protein phosphatases (Pamerter et al., 2007, 2008a,b; Shin et al., 2005). In addition, we have recently shown that mitochondrial ATP-sensitive K⁺ channels (mK_{ATP}) can regulate the anoxic decrease in NMDAR activity (Pamerter et al., 2007, 2008a,b) and are also sensitive to PKC and PKG via mechanisms that may involve G_i protein cascades (Costa et al., 2005; Kis et al., 2003). Thus, anoxic turtle NMDAR activity may be regulated by changes in G_i protein signaling cascades that occur independently of adenosine receptor activation.

The aims of this paper are to examine: (1) the effects of adenosine and A₁R manipulation on whole-cell NMDAR peak currents during normoxia and a normoxic to anoxic transition; (2) the role of inhibitory G_i proteins in the adenosine- and anoxia-mediated change in NMDAR peak currents; and (3) whether or not the adenosine-mediated change in NMDAR activity acts via the same pathway as mK_{ATP} activation.

2. Results

Changes in NMDAR currents during normoxic to anoxic transitions have been described previously but are repeated here for reproducibility and statistical comparison. NMDAR currents did not change in magnitude during 40 min of normoxic recordings, ranging from 850.4±87.5 to 937.3±74.8 pA (Figs. 1A and B, n=8). Conversely, currents decreased by 45.5±8.6 and 51.3±3.9% at 20 and 40 min of anoxia, respectively (Figs. 2A and B, n=8). Anoxic NMDAR currents recovered to control levels following 20 min of re-oxygenation (data not shown).

During normoxia, application of 250 μM adenosine resulted in an anoxic-like decrease in NMDAR currents of 47.2±6.2 and 57.1±11.3% following 20 and 40 min of adenosine perfusion,

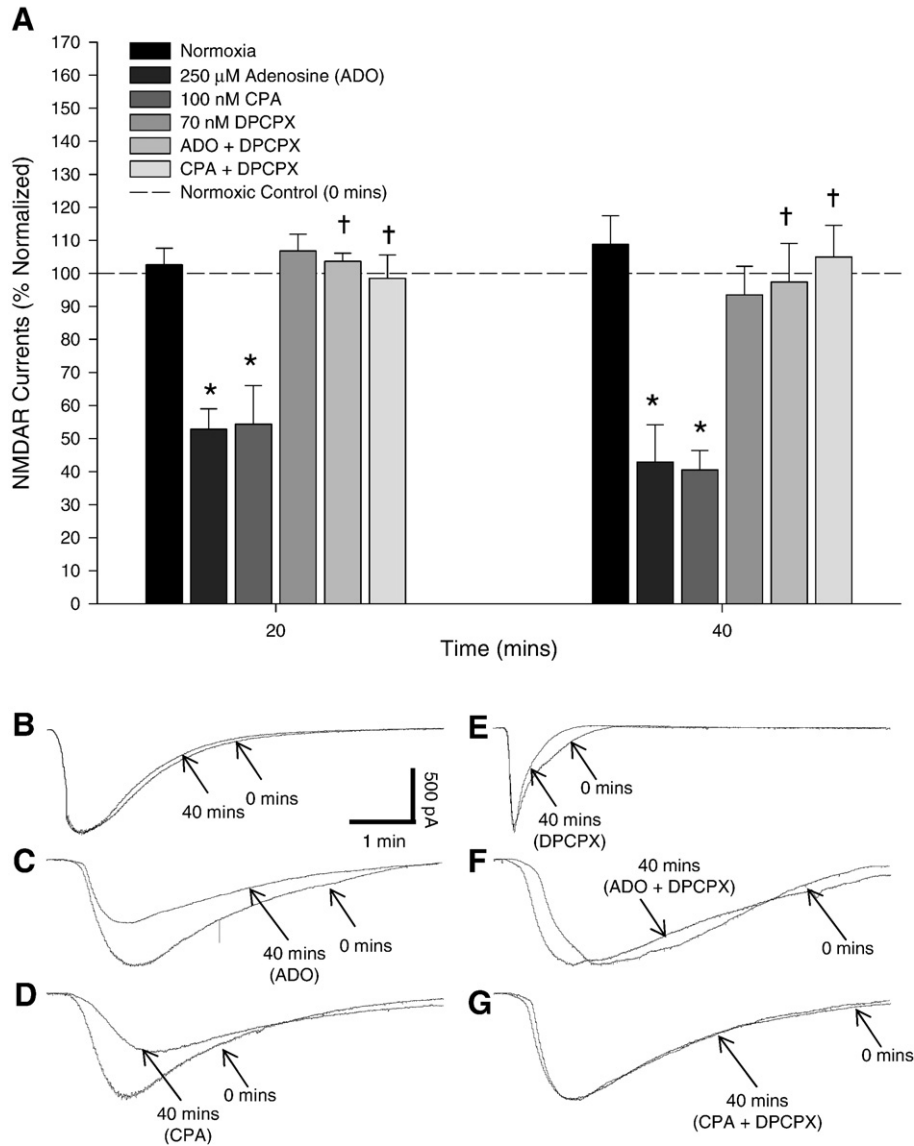


Fig. 1 – A_1 receptors regulate normoxic NMDAR currents. (A) Summary of changes in peak NMDAR currents during normoxia and following treatment with adenosine receptor modulators. NMDAR currents following treatment ($t=20, 40$ min) were normalized to control recordings from the same cell ($t=0$ min, shown as dashed line). Data are expressed as mean \pm SEM. Asterisks indicate data significantly different from normoxic controls. Daggers indicate significant difference from ADO or CPA perfusion alone. Significance was assessed at $P < 0.05$. (B–G) Raw data traces of whole-cell NMDAR currents during treatment with (B) a sham switch, (C) the adenosine receptor (A_1 R) agonists adenosine (ADO) or (D) N6-cyclopentyladenosine (CPA), (E) the A_1 R antagonist 8-cyclopentyl-1,3-dimethylxanthine (DPCPX), or (F–G) combined application of A_1 R agonists and DPCPX.

respectively (Figs. 1A and C, $n=8$). Similarly, perfusion of the A_1 R agonist CPA also decreased NMDAR currents by 45.6 ± 11.7 and $59.4 \pm 5.9\%$ at 20 and 40 min of CPA perfusion, respectively (Figs. 1A and D, $n=8$). The specificity of these responses to A_1 R activation was confirmed by the presence of the A_1 R antagonist DPCPX, which blocked the effect of the two agonists (Figs. 1A, F and G, $n=8$ for ADO plus DPCPX, $n=7$ for CPA plus DPCPX). DPCPX perfusion alone had no effect on normoxic NMDAR currents (Figs. 1A and E, $n=4$).

To examine the role of adenosine in the anoxic decrease in NMDAR activity, anoxic cells were concomitantly treated with adenosine or CPA. The anoxic decrease in NMDAR activity

with simultaneous adenosine or CPA application did not differ from anoxic treatment alone (Figs. 2A, C and D, $n=5$ for each). Blockade of A_1 Rs with DPCPX during anoxia did not abolish the anoxia-mediated decrease in NMDAR currents, which were depressed by 28.6 ± 6.8 and $53.4 \pm 10.2\%$ at 20 and 40 min of anoxic perfusion, respectively (Figs. 2A and E, $n=5$ for each).

Adenosine receptors are G protein-coupled receptors and adenosine–ligand binding results in dissociation of an inhibitory G_i protein subunit. In another set of experiments cortical sheets were incubated overnight at 4°C in 400 nM pertussis toxin (PTX), an inhibitor of G_i protein activation. In tissues pre-treated with PTX the anoxic decrease in NMDAR activity was

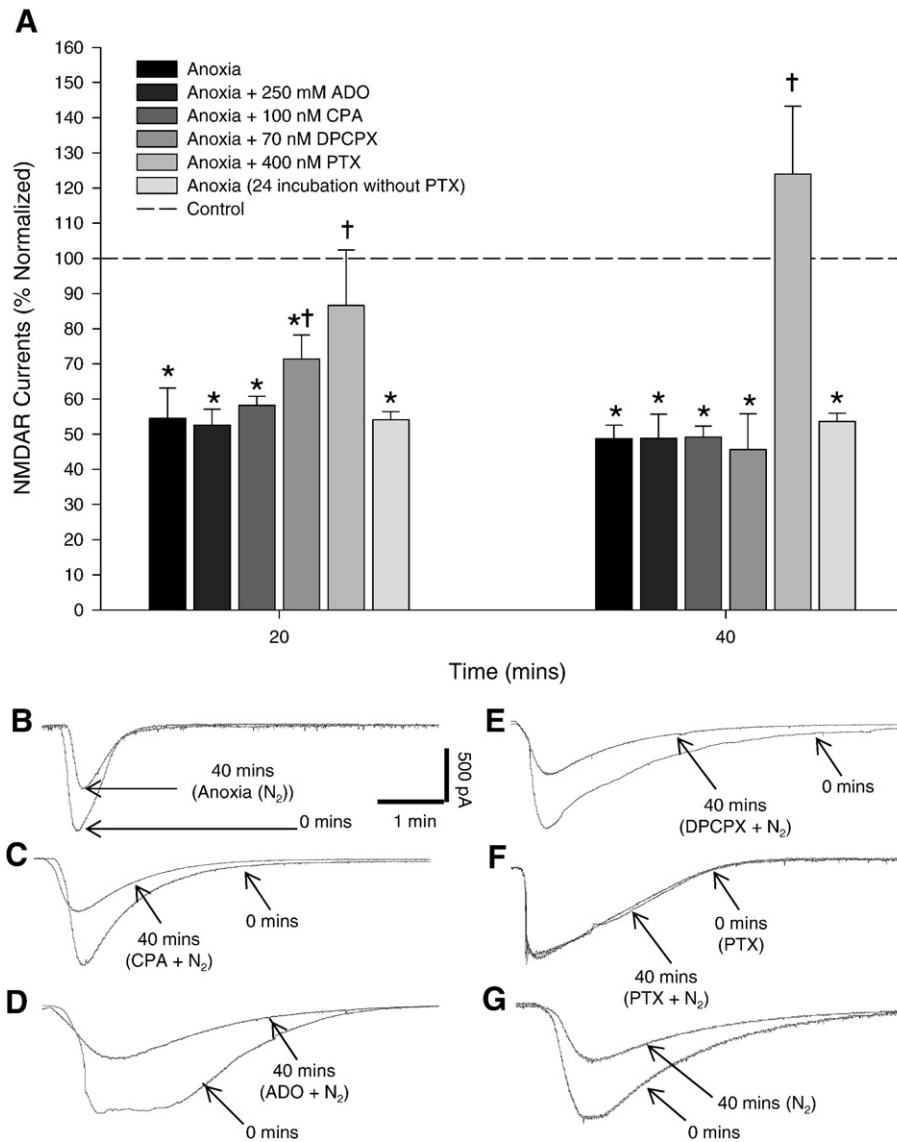


Fig. 2 – G_i proteins but not A₁ receptors mediate the anoxic decrease in NMDAR currents. (A) Summary of changes in peak NMDAR currents during normoxia and following treatment with adenosine receptor modulators or following pre-incubation with the G_i protein inhibitor pertussis toxin (PTX). Anoxic NMDAR currents following treatment (t=20, 40 min) were normalized to control recordings from the same cell (t=0 min, shown as dashed line). Data are expressed as mean ± SEM. Asterisks indicate data significantly different from normoxic controls. Daggers indicate significant difference from anoxic controls. Significance was assessed at P<0.05. (B–F) Raw data traces of whole-cell NMDAR currents following normoxic to anoxic transitions (B) or anoxic treatment combined with (C) CPA or (D) ADO, (E) DPCPX, (F) PTX, or (G) in tissues pre-incubated for >24 h without PTX.

not observed (Figs. 2A and F, n=5). The absolute magnitude of NMDAR peak currents recorded from normoxic slices incubated in PTX was 1025.2 ± 125.0 pA and was not significantly different from sheets that had not been incubated in PTX (data not shown). Additionally, incubation of sheets in normoxic saline for 24 h at 4 °C had no effect on peak NMDAR currents, which had a mean amplitude of 959 ± 54.6 pA (n=7, raw data not shown). Finally, when control pre-incubated sheets were exposed to anoxic perfusion, NMDAR currents decreased $53.6 \pm 2.4\%$, which was not significantly different from the anoxic decrease observed in freshly isolated cortical sheets (Figs. 2A and G, n=7).

G proteins, mK_{ATP} channels and changes in [Ca²⁺]_c mediate the decrease in turtle NMDAR activity during anoxia (Bickler et al., 2000; Pamenter et al., 2007, 2008a,b). Therefore, we hypothesized that adenosine or activation of the A₁R during normoxia may increase the activity of G_i proteins, which decreases [cAMP] and triggers anoxia-like reductions in NMDAR currents during normoxia via mK_{ATP} channels and changes in [Ca²⁺]_c. Similar to the anoxic decrease, the CPA-mediated decrease in NMDAR activity was prevented by PTX pre-incubation (Figs. 3A and B, n=5). Furthermore, the effects of both adenosine (n=13) and CPA (n=11) were reversed by the mK_{ATP} antagonist SHD (Figs. 3A, C and D). In addition, the presence of the Ca²⁺-chelator BAPTA in

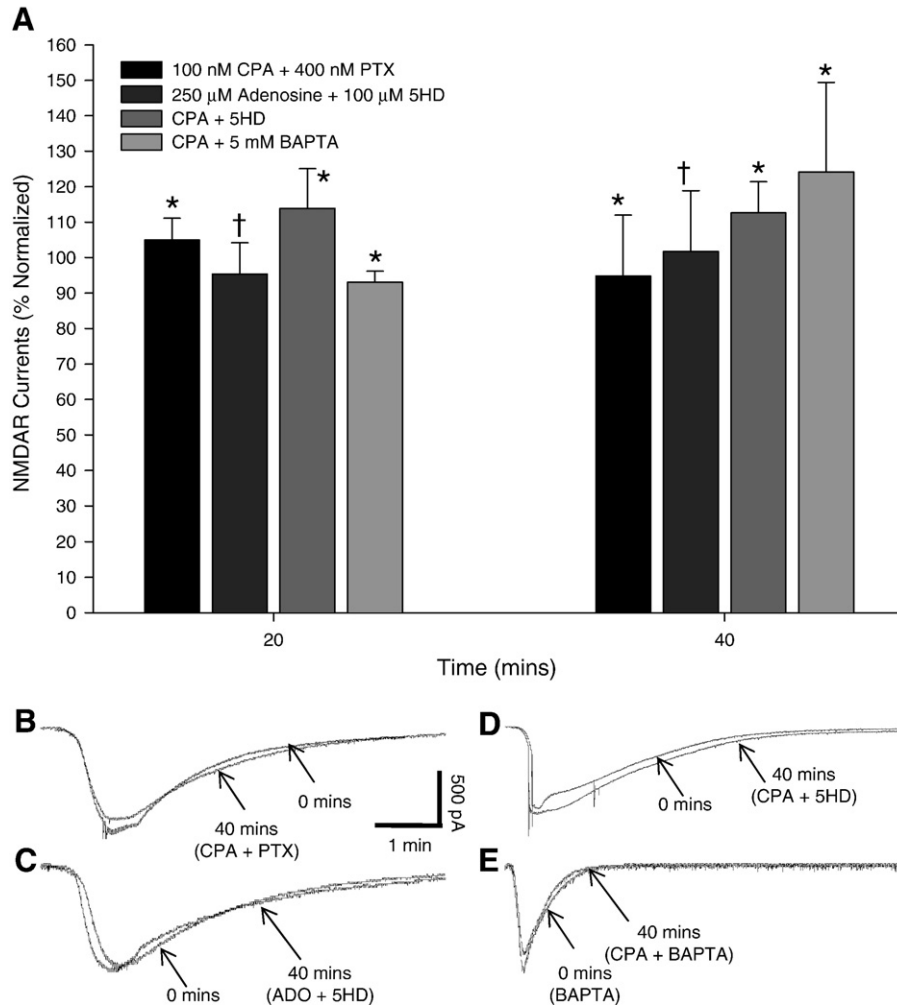


Fig. 3 – A_1 receptor-mediated NMDAR depression functions via a pathway involving G_i proteins, mK_{ATP} channels and changes in $[Ca^{2+}]_c$. (A) Summary of changes in peak NMDAR currents during normoxia and following treatment with adenosine receptor activators and co-treatment with G_i or ATP-sensitive K^+ channel (mK_{ATP}) inhibitors or with Ca^{2+} chelation. NMDAR currents following treatment ($t=20, 40$ min) were normalized to control recordings from the same cell ($t=0$ min). Data are expressed as mean \pm SEM. Asterisks indicate data significantly different from normoxic CPA controls shown in Fig. 1. Daggers indicate data significantly different from normoxic adenosine controls shown in Fig. 1 ($P < 0.05$). (B–E) Raw data traces of whole-cell NMDAR currents following co-perfusion of adenosine agonists and (B) PTX, (C–D) the mK_{ATP} agonist 5-hydroxydecanoic acid (5HD), or (E) BAPTA.

the recording electrode prevented the CPA-mediated decrease in NMDAR activity (Figs. 3A and E, $n=3$).

3. Discussion

Our previous results examining the role of adenosine receptors in the regulation of anoxic turtle cortical NMDAR activity are conflicting. Previously it was reported that A_1R antagonism prevented the anoxic change in NMDAR P_{open} but not the anoxic change in NMDAR-mediated Ca^{2+} flux (Buck and Bickler, 1998, 1995). This discrepancy may be explained by experimental design. In examining P_{open} , Buck and Bickler (1995) pre-incubated tissue with the A_1R antagonist 8-phenyltheophylline (8-PT) and then exposed cortical slices to either anoxia or adenosine perfusion. Changes in P_{open} were not observed in either of these experi-

ments; however, examination of the mean P_{open} of these treatment groups suggests that P_{open} was depressed in pre-incubated tissues. In fact, the observed average P_{open} of pre-incubated tissue was similar to values recorded from either anoxia or adenosine perfused neurons (Buck and Bickler, 1998). This depression is not likely due to the effect of tissue incubation since in our control experiments, the anoxic depression in whole-cell NMDAR currents persisted in cortical sheets incubated for >24 h in normoxic aCSF. P_{open} may have been depressed in pre-incubated tissue due to non-specific actions of 8-PT, particularly as P_{open} was depressed in both groups pre-treated with 8-PT, and not depressed in the four non pre-treated groups.

Our present experimental design offers a direct comparison of the effect of A_1R blockade during anoxia because we used a paired design and measured control and treatment recordings in the same cell. In this study, adenosine attenuated NMDAR

currents via activation of A_1 Rs and this attenuation was prevented by A_1 R blockade, G_i protein inhibition, mK_{ATP} blockade, or by chelation of intracellular Ca^{2+} . A_1 Rs appear to play a limited role in the anoxic depression of NMDAR currents. During early anoxia (20 min), A_1 R antagonism reduced the anoxic decrease in NMDAR currents by ~20%; however, by 40 min of anoxia A_1 R antagonism failed entirely to abolish the decrease. Conversely, G_i protein inhibition completely abolished the decrease of NMDAR currents at both 20 and 40 min of anoxic perfusion. These data support a role for A_1 R-mediated NMDAR regulation during normoxia but only a limited role during early anoxia. The normoxic and prolonged anoxic pathways are likely different but converge onto a common signaling pathway involving G_i protein-coupled messaging.

If this were the case, activation of adenosine-mediated G_i protein-coupled messaging during normoxia would suppress NMDAR activity via G_i protein-based mechanisms that are otherwise activated by anoxia. Thus, blockade of adenosine receptors during anoxia would have no effect on NMDAR currents since the G_i protein-based mechanism initiated during anoxia is already active. Our results from cortical sheets pre-incubated with the G_i inhibitor PTX support this scenario. We report that anoxia and the CPA-mediated decrease in NMDAR currents are prevented by PTX pre-incubation, suggesting that both mechanisms rely on G_i protein signaling. In turtle brain, adenosine increases rapidly following anoxic perfusion, but then oscillates during prolonged anoxia (Lutz and Kabler, 1997). As such, it is not an appropriate regulator of sustained anoxic NMDAR depression. However, elevations in adenosine may partially contribute to depression in NMDAR activity during early anoxia since DPCPX reduced the anoxic decrease in NMDAR activity at 20 but not 40 min of anoxia in our experiments.

Since PTX, but not DPCPX, completely abolished the anoxic decrease in NMDAR activity at both 20 and 40 min, it is likely that the anoxic depression involves the activity of G_i protein-coupled receptors other than A_1 Rs. One such receptor in the turtle brain is the delta opioid receptor (DOR), whose expression is significantly higher and more ubiquitously distributed in turtle brain than in anoxia-sensitive rat brain (Xia and Haddad, 2001). Furthermore, DOR agonism is neuroprotective against ischemia in mammalian brain and prevents toxic elevations in Ca^{2+} that are characteristic of ECD (Chao et al., 2007a,b). Also, there is an inverse relationship between DORs and NMDARs whereby activation of one receptor leads to inhibition of the other, and vice versa (Cao et al., 1997; Suzuki et al., 2000; Wang and Mokha, 1996; Zhang et al., 1996).

Although A_1 Rs are not involved in the anoxic regulation of whole-cell NMDAR activity in the turtle cortex, adenosine still plays a critical neuroprotective role in the anoxic turtle brain. Since blockade of A_1 Rs during anoxia results in increased neuronal death in turtle brain (Milton et al., 2007; Perez-Pinzon et al., 1993), A_1 Rs must regulate other critical mechanisms of anoxia tolerance. One possible mechanism of adenosine-mediated protection is via the regulation of glutamate release during prolonged anoxia. Reductions in glutamate release reduce AMPAR and NMDAR activities independent of post-synaptic receptor modulation, thereby decreasing neuronal susceptibility to excitotoxic events (Milton and Lutz, 2005; Milton et al., 2002; Thompson et al., 2007). Alternatively, adenosine may

depress electrical activity exclusive of glutamatergic regulation (Materi et al., 2000; Mei et al., 1994; Obrietan et al., 1995; Rezvani et al., 2007). For example, adenosine receptor activity prevented increased excitatory synaptic events in the turtle basal optic nucleus (Ariel, 2006). Adenosine also plays a role in the regulation of cerebral blood flow (CBF) during anoxic episodes. Adenosine causes vasodilation of cerebral blood vessels, resulting in increased blood flow and enhanced delivery of glycolytic substrate (Collis, 1989). In the anoxic turtle brain, CBF increases nearly 2-fold, while adenosine perfusion during normoxia increases CBF nearly 4-fold. Furthermore, both of these increases were prevented by A_1 R blockade (Hylland et al., 1994). Turtle brain undergoes a remarkable suppression of energy-utilizing mechanisms during anoxia (Bickler and Buck, 2007; Milton and Prentice, 2007). However, neuronal activity is not completely shut off and neurons are thus dependent on glycolytic metabolism for energy production (Doll et al., 1994; Hochachka et al., 1996). Therefore delivery of glycolytic substrate to the brain is critical to surviving anoxic insults and an increase in CBF may be neuroprotective in anoxic turtle brains.

In conclusion, we demonstrate that activation of A_1 Rs during normoxia depresses turtle cortical NMDAR currents via a pertussis toxin-sensitive mechanism that also involves mK_{ATP} channels and changes in $[Ca^{2+}]_c$. During anoxia, similar decreases in NMDAR activity are observed that are mediated by the same downstream signaling components (G_i proteins) but appear to occur independently of A_1 R activation. These data resolve previous discrepancies regarding the role of adenosine in NMDAR regulation in the anoxic turtle cortex and indicate that the mechanisms of anoxic neuroprotection mediated by adenosine likely do not involve direct regulation of NMDAR activity.

4. Experimental procedures

4.1. Ethics approval

This study was approved by the University of Toronto Animal Care committee and conforms to the Guide to the Care and Use of Experimental Animals, Volume 2 as determined by the Canadian Council on Animal Care regarding relevant guidelines for the care of experimental animals. Adult turtles were obtained from Niles Biological Inc. (Sacramento, CA, USA).

4.2. Dissection and whole-cell patch-clamp recordings

All experiments were conducted at a room temperature of 22 °C. Turtles were decapitated and whole brains were rapidly excised from the cranium within 30 s of decapitation. Cortical sheets were isolated from the whole brain in ice-cold saline as described elsewhere (Blanton et al., 1989), and then placed in a RC-26 chamber with a P1 platform (Warner Instruments, CT, USA). Cortical sheets were bathed in artificial turtle cerebrospinal fluid (aCSF; in mM: 107 NaCl, 2.6 KCl, 1.2 $CaCl_2$, 1 $MgCl_2$, 2 NaH_2PO_4 , 26.5 $NaHCO_3$, 10 glucose, 5 imidazole, pH 7.4; osmolarity 280–290 mOsm). The chamber was gravity perfused at a rate of 2–3 ml/min. Normoxic aCSF was gassed with 95% O_2 /5% CO_2 and anoxic aCSF with 95% N_2 /5% CO_2 . To maintain anoxic conditions, perfusion tubes from IV bottles were double jacketed

and the outer jacket gassed with 95%N₂/5%CO₂. The anoxic aCSF reservoir was bubbled for 30 min before experiments. A plastic cover with a hole for the recording electrode was placed over the perfusion chamber and the space between the fluid surface and the cover was gently gassed with 95%N₂/5%CO₂. Throughout the entire anoxic experiment, aCSF was constantly gassed with this N₂/CO₂ mixture. The partial pressure of oxygen (PO₂) in the recording chamber decreased from approximately 610 mmHg PO₂ to 0.5 mmHg PO₂ (anoxia) within 5 min, which is the limit of detection for the PO₂ electrode and not different from that in the N₂/CO₂ bubbled reservoir. PO₂ levels were maintained at this level for the duration of anoxic experiments (data not shown).

Whole-cell recordings were performed using 2–4 MΩ electrodes containing the following (in mM: 8 NaCl, 0.0001 CaCl₂, 10 NaHEPES, 110 Kgluconate, 1 MgCl₂, 0.3 NaGTP, and 2 NaATP, adjusted to pH 7.4). Cell-attached 5–20 GΩ seals were obtained using the blind-patch technique described elsewhere (Blanton et al., 1989). Whole-cell patches were obtained by applying a brief suction and data were collected at 2 kHz using an Axopatch-1D amplifier, a CV-4 headstage, and a TL-1 DMA interface (Axon Instruments, CA, USA) and then digitized and stored on a computer using Clampex 7 software (Axon Instruments, CA, USA). Typical access resistance was 10–30 MΩ. Access resistance was determined prior to each measurement of electrical activity (see below) and patches were discarded if access resistance changed by more than 20% during the course of an experiment.

Whole-cell peak NMDAR currents were recorded for up to 2 h from pyramidal neurons as described elsewhere (Shin and Buck, 2003), and neurons of other types were discarded. Normoxic experiments consisted of an O₂/CO₂ aCSF perfusion as described above. A fast-step perfusion system (VC-6 perfusion valve controller and SF-77B fast-step perfusion system, Warner Instruments, CT, USA) was used to deliver 1 μM tetrodotoxin (TTX) and 300 μM NMDA. Prior to each recording cortical sheets were perfused with TTX for 5 min to prevent action potentials. Cells were then voltage clamped at –70 mV and NMDA was applied until a current was elicited (3–10 s, depending on the proximity of the perfusion system to the patched neuron). This NMDA application time was used for all recordings from the same neuron within a single experiment. The initial peak NMDA current was set to 100% and subsequent peak NMDA currents were normalized to this value. For anoxic and pharmacological experiments, NMDA was initially applied to cortical sheets in normoxic aCSF, and the evoked whole-cell current was set to 100% (t=0 min), cortical sheets were then exposed to anoxic aCSF or aCSF containing specific receptor modulators for 40 min. NMDA-evoked peak currents were monitored at 20-minute intervals following the change in aCSF. Cells were then reperfused with control normoxic aCSF for 40 min and NMDA-evoked peak currents were monitored at 20-minute intervals following re-oxygenation. NMDAR currents are not inhibited by the concentration of Mg²⁺ used in these experiments (Shin and Buck, 2003).

4.3. Chemicals

All chemicals were obtained from Sigma Chemical Co. (Oakville, ON, Canada). The chemicals were dissolved in the bulk perfusate except where specified otherwise. Adenosine A₁ recep-

tors were activated by either 250 μM adenosine or the A₁ agonist N⁶-cyclopentyladenosine (CPA, 100 nM), and blocked by the A₁ specific antagonist 8-cyclopentyl-1,3-dimethylxanthine (DPCPX, 70 nM). G proteins were blocked by the G_i/G_o inhibitor pertussis toxin (PTX). Cortical sheets were incubated in normoxic saline containing 400 nM PTX for 18 h prior to experimentation. mK_{ATP} were blocked by the mK_{ATP}-specific antagonist 5-hydroxydecanoic acid (5HD, 100 μM). For experiments involving calcium, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, 5 mM) was included in the recording electrode solution to chelate intracellular calcium. CPA and DPCPX were initially dissolved in dimethylsulphonic acid (DMSO), and then placed in aCSF not exceeding 1% v/v. Vehicle application alone did not affect NMDA-evoked currents (data not shown).

4.4. Statistical analysis

NMDAR whole-cell current data were analyzed following root arcsine transformation using two-way ANOVA with a Student–Newman–Keuls (all pair wise) post-hoc test to compare within and against treatment and normoxic values. Significance was determined at P<0.05, and all data are expressed as the mean ± SEM (standard error of mean).

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