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δ-Opioid receptor antagonism induces NMDA receptor-dependent excitotoxicity in anoxic turtle cortex

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SUMMARY

δ-Opioid receptor (DOR) activation is neuroprotective against short-term anoxic insults in the mammalian brain. This protection may be conferred by inhibition of *N*-methyl-D-aspartate receptors (NMDARs), whose over-activation during anoxia otherwise leads to a deleterious accumulation of cytosolic calcium ($[Ca^{2+}]_c$), severe membrane potential (E_m) depolarization and excitotoxic cell death (ECD). Conversely, NMDAR activity is decreased by ~50% with anoxia in the cortex of the painted turtle, and large elevations in $[Ca^{2+}]_c$, severe E_m depolarization and ECD are avoided. DORs are expressed in high quantity throughout the turtle brain relative to the mammalian brain; however, the role of DORs in anoxic NMDAR regulation has not been investigated in turtles. We examined the effect of DOR blockade with naltrindole (1–10 μmol l^{-1}) on E_m, NMDAR activity and $[Ca^{2+}]_c$ homeostasis in turtle cortical neurons during normoxia and the transition to anoxia. Naltrindole potentiated normoxic NMDAR currents by 78±5% and increased $[Ca^{2+}]_c$ by 13±4%. Anoxic neurons treated with naltrindole were strongly depolarized, NMDAR currents were potentiated by 70±15%, and $[Ca^{2+}]_c$ increased 5-fold compared with anoxic controls. Following naltrindole washout, E_m remained depolarized and $[Ca^{2+}]_c$ became further elevated in all neurons. The naltrindole-mediated depolarization and increased $[Ca^{2+}]_c$ were prevented by NMDAR antagonism or by perfusion of the G_i protein agonist mastoparan-7, which also reversed the naltrindole-mediated potentiation of NMDAR currents. Together, these data suggest that DORs mediate NMDAR activity in a G_i-dependent manner and prevent deleterious NMDAR-mediated $[Ca^{2+}]_c$ influx during anoxic insults in the turtle cortex.

Key words: anoxic depolarization, channel arrest, excitotoxic cell death.

INTRODUCTION

Mammalian neurons are acutely sensitive to anoxia and rapidly undergo excitotoxic cell death (ECD) when deprived of oxygen. ECD is characterized by increased glutamate release, which leads to over-activation of *N*-methyl-D-aspartate receptors (NMDARs), toxic accumulation of intracellular calcium ($[Ca^{2+}]_c$), and a severe loss of membrane potential (E_m) termed anoxic depolarization (AD) (Choi, 1994; Lundberg and Oscarsson, 1953). ECD and AD can be avoided and mammalian neurons can be made relatively anoxia tolerant *via* an inducible neuroprotective mechanism known as hypoxic preconditioning (HPC), whereby pretreatment with a tolerable hypoxic insult confers neuroprotection against subsequent, otherwise neurotoxic insults (Murry et al., 1986; Schurr et al., 1986). The specific mechanism of this inducible protection is poorly understood although numerous receptors have been implicated, including δ-opioid receptors (DORs).

DORs are a class of inhibitory G protein (G_i)-coupled receptor whose activation is neuroprotective against hypoxic or ischemic insults in mammalian neurons (Chao et al., 2007b; Zhang et al., 2002; Zhang et al., 2000). Recently, DORs have also been linked to HPC-mediated neuroprotective mechanisms. In HPC-treated animals, DOR mRNA and protein titers are elevated concomitantly with increased neuroprotection, while a DOR antagonist abolishes neuroprotection. In addition, DOR antagonism induces cell death in normoxic rat cortical neurons and accelerates anoxia-induced cell death (Zhang et al., 2002). This finding is particularly important as it suggests that a tonic background level of DOR activation is critical to neuronal survival in non-pathological environments as well as during pathological events.

Despite strong evidence supporting a role for DORs in neuroprotection against anoxic insults, the mechanism for this is unclear. Interestingly, research into the effects of NMDAR pharmacological agents on the mechanism of opioid dependency provided evidence for a cross-talk mechanism between DORs and NMDARs such that activation of DORs reduces NMDAR activity and *vice versa* (Cao et al., 1997; Wang and Mokha, 1996). Since over-activation of NMDARs is central to ECD and AD following ischemic insult, and since DOR activation provides neuroprotection against such insults, it is reasonable to suspect that DOR-mediated regulation of NMDAR activity is neuroprotective.

The western painted turtle (*Chrysemys picta bellii* Schneider 1783) survives months of acute anoxic exposure at 3°C and days of anoxia at 25°C without apparent detriment (Jackson, 1968; Musacchia, 1959). In the anoxic turtle cortex, NMDAR activity is decreased, large increases in $[Ca^{2+}]_c$ are avoided and ECD and AD are not observed (Bickler et al., 2000). With the emerging role for DORs in mammalian HPC models, it is interesting to note that turtle brain expresses very high endogenous levels of DORs in comparison with rat brain and that, unlike in rat, DORs are abundantly distributed throughout all regions of the turtle brain (Xia and Haddad, 2001). Given the functional connection between DORs and NMDARs in mammalian brain and the widespread abundance of DORs in turtle brain, we hypothesized that DOR activity may be involved in the regulation of turtle cortical NMDAR activity during anoxia. The

aim of this study was to examine the effect of DOR blockade on turtle cortical neuron electrical activity, whole-cell NMDAR peak current activity and $[Ca^{2+}]_c$ homeostasis during normoxia and during transition to anoxia with recovery.

MATERIALS AND METHODS

Dissection and whole-cell patch-clamp recordings

This study conforms to the University of Toronto Animal Care committee 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 (Sacramento, CA, USA). Methods of cortical sheet isolation, artificial cerebrospinal fluid (ACSF) composition, and equipment setup for anoxic whole-cell NMDAR current and Ca^{2+}-imaging experiments are described elsewhere (Blanton et al., 1989; Pamenter et al., 2008d). All experiments were conducted at a room temperature of 22°C. Whole-cell recordings were performed using 3–6 MΩ electrodes containing the following (in mmol l^{-1}): 8 NaCl, 0.0001 $CaCl_2$, 10 NaHepes, 110 potassium gluconate, 1 $MgCl_2$, 0.3 Na_2GTP and 2 Na_2ATP (adjusted to pH 7.4). The bath reference electrode was a Ag–AgCl junction. 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 brief suction following seal formation. Typical access resistance (R_a) was 10–30 MΩ. R_a was determined prior to each measurement and patches were discarded if R_a changed by more than 20% during the course of an experiment. Data were collected at 2 kHz using an Axopatch-1D amplifier, a CV-4 headstage and a digidata 1200 using Clampex 7 software (Axon Instruments, Union City, CA, USA). The liquid junction potential was assessed as ~10 mV and all data have been corrected for this value (raw data traces are unaltered). All chemicals were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Fluorescent probes were obtained from Molecular Probes (Eugene, OR, USA).

Experimental design

Cortical neurons were perfused with pharmacological modifiers in the bulk perfusate as specified in the Results section. DORs were blocked with 1–10 μmol l^{-1} naltrindole (Zhang et al., 2000). NMDARs were blocked with 2-amino-5-phosphonopentanoic acid (APV: 70 nmol l^{-1}). G_i proteins were stimulated with mastoparan-7 (MP7: 0.1–1 μmol l^{-1}). MP7 is oxygen sensitive and was prepared under nitrogen and then injected directly into ACSF pre-gassed with 95% N_2/5% CO_2. Baseline spontaneous electrical activity, NMDAR activity and $[Ca^{2+}]_c$ fluorescence were measured for >10 min and cortical sheets were exposed to anoxic ACSF or ACSF containing pharmacological modulators for a minimum of 40 min and then reperfused for 20–40 min with normoxic ACSF. Electrical activity and $[Ca^{2+}]_c$ were recorded throughout, while NMDAR currents were elicited at 0 and 10 min of control perfusion and then at 20 min intervals following the switch to treatment ACSF by puffing 300 μmol l^{-1} NMDA onto cortical sheets. $[Ca^{2+}]_c$ changes were assessed using fura-2 excited at 340 and 380 nm at 10 s intervals to limit photo-bleaching. Fluorescence emissions above 510 nm were isolated using an Olympus DM510 dichroic mirror and fluorescence measurements were acquired (515–530 nm). $[Ca^{2+}]_c$ was calculated as described elsewhere (Buck and Bickler, 1995).

Statistical analysis

Changes in E_m were assessed by comparing the average E_m from 5 min segments. For each fura-2 experiment, 25 neurons were chosen at random and the average change in fluorescence 5 min after each perfusion switch was used for statistical comparison. Significance was determined using a one-way repeated ANOVA followed by Tukey's *post-hoc* test. NMDAR current data were normalized and analyzed using two-way ANOVA with a Student–Neuman–Keuls *post-hoc* test. Significance was determined at $P<0.05$, and all data are expressed as the mean ± s.e.m. (standard error of the mean).

RESULTS

During normoxia, E_m did not change significantly over 1 h of recording in any neuron (Fig. 1A; Fig. 2; N=8), while anoxic perfusion resulted in a rapid depolarization of ~8 mV that was reversed following reoxygenation (Fig. 1B; Fig. 2; N=15). The anoxic changes in turtle NMDAR activity and $[Ca^{2+}]_c$ homeostasis are well documented but these experiments were repeated here for statistical

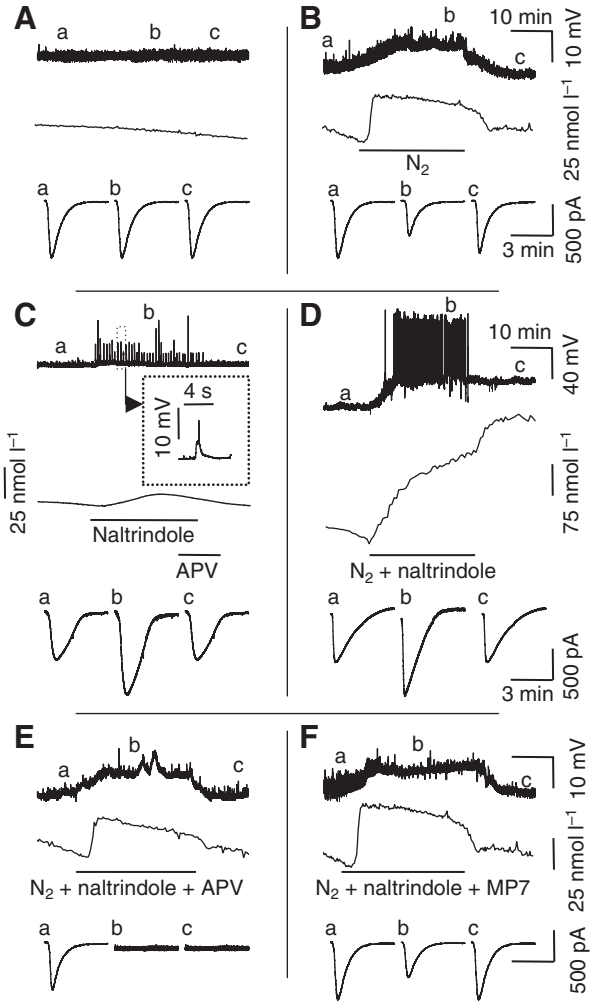

Fig. 1. Neuronal responses to naltrindole during anoxia. For each panel the top trace (membrane potential, E_m) and the middle trace (change in cytosolic $[Ca^{2+}]$, $\Delta[Ca^{2+}]_c$) correspond to the same treatment regime, as indicated by the solid black bars. The whole-cell NMDAR currents (bottom trace) were recorded at the time points indicated by a, b or c on the E_m trace within the same panel. All experiments were conducted on individual cortical sheets exposed to (A) normoxia, (B) anoxia, (C) normoxia plus naltrindole, (D) anoxia plus naltrindole, (E) anoxia plus naltrindole in the presence of the NMDAR antagonist 2-amino-5-phosphonopentanoic acid (APV), or (F) anoxia plus naltrindole in the presence of the G_i agonist mastoparan-7 (MP7).

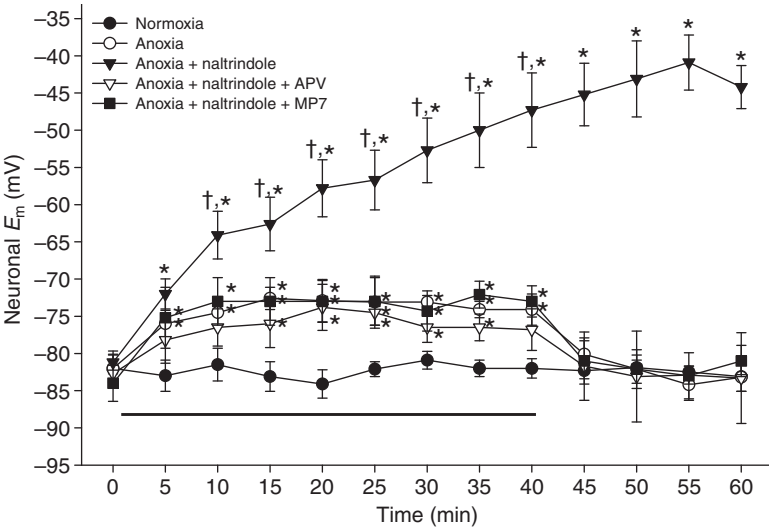

Fig. 2. Naltrindole enhances anoxic neuronal depolarization *via* G_i protein inhibition. Summary of E_m changes from cortical neurons during treatment. E_m was assessed as the mean of sequential 5 min recording segments. Data are expressed as means ± s.e.m. *Data significantly different from normoxic controls; †data significantly different from anoxic controls. Significance was assessed at $P<0.05$. The bar indicates the duration of the treatment indicated in the key.

comparisons (Bickler et al., 2000; Pamenter et al., 2008d; Shin and Buck, 2003). During 90 min of control recordings, normoxic NMDAR peak currents did not change significantly, ranging from 986±120 to 1087±73 pA, and $[Ca^{2+}]_c$ was determined to be 142±8 nmol l^{-1} (Fig. 1A; Figs 3 and 4; N=5 for each). Anoxic perfusion induced a 50±7% and 53±9% reduction in NMDAR activity at 20 and 40 min of anoxia, respectively (Fig. 1B; Fig. 3; N=8). Also, $[Ca^{2+}]_c$ increased with anoxia by ~40% to 199±15 nmol l^{-1} (Fig. 1B; Fig 4; N=5). Following normoxic reperfusion, E_m, NMDAR currents and $[Ca^{2+}]_c$ returned to control levels.

Naltrindole perfusion during normoxia did not lead to significant sustained E_m depolarization; however, large discrete depolarizing events were observed at a frequency of 0.06±0.01 Hz (Fig. 1C, inset; Fig. 2; N=20). APV perfusion abolished these depolarizing currents, suggesting they were mediated by NMDARs. Naltrindole potentiated normoxic NMDAR peak currents by 49±6% and 78±4% following 20 and 40 min treatment, respectively (Fig. 1C; Fig. 3; N=6), and increased $[Ca^{2+}]_c$ by 13±4% (Fig. 1C; Fig. 4; N=6). Both these changes were reversed by washout of naltrindole. APV also abolished the naltrindole-mediated increase in $[Ca^{2+}]_c$ in normoxic controls, indicating the increase was due to NMDAR-mediated calcium influx (Fig. 4; N=5).

During anoxia, naltrindole resulted in hyper-excitability, extended depolarization and, in 7 out of 23 experiments (31%), terminal loss of E_m (Fig. 1D; Fig. 2; N=23). In all 23 neurons, the extended depolarization was not reversed by reoxygenation, suggesting impaired neuronal viability following anoxia. The depolarization may have been due to increased NMDAR activity because naltrindole prevented the anoxic decrease in NMDAR peak currents. In fact, naltrindole potentiated NMDAR currents by 44±19% and 70±15% following 20 and 40 min of anoxic perfusion (Fig. 1D; Fig. 3; N=6). This increase resembles the potentiation of turtle NMDAR currents by naltrindole during normoxia but is striking compared with the ~50% decrease in NMDAR activity normally observed in anoxic cortical neurons. Concomitant with the increase in NMDAR current magnitude and enhanced anoxic depolarization, naltrindole treatment during anoxia resulted in significantly greater increases in $[Ca^{2+}]_c$ compared with anoxic controls. In these neurons $[Ca^{2+}]_c$ increased 212±16% to 302±22 nmol l^{-1}; and unlike in control anoxic recordings, $[Ca^{2+}]_c$ continued to increase following washout with normoxic naltrindole-free saline to 544±56 nmol l^{-1}, a 382% increase over control $[Ca^{2+}]_c$ and ~350% greater than $[Ca^{2+}]_c$ in control anoxic experiments following reoxygenation (Fig. 1D; Fig. 4; N=5).

We hypothesized that the electrical hyper-excitability and loss of E_m observed in most anoxic turtle cortical neurons treated with naltrindole may be due to over-activation of NMDARs and subsequent NMDAR-mediated excitotoxic elevations in $[Ca^{2+}]_c$. Blockade of NMDARs with APV prevented whole-cell NMDA-evoked currents (Fig. 1E), and had no effect on $[Ca^{2+}]_c$ or E_m (data not shown). Perfusion of APV during anoxia prevented naltrindole-mediated electrical hyper-excitability and extended depolarization (Fig. 1E; Fig. 2; N=7). The electrical response of anoxic neurons treated with naltrindole and APV resembled that of the response to

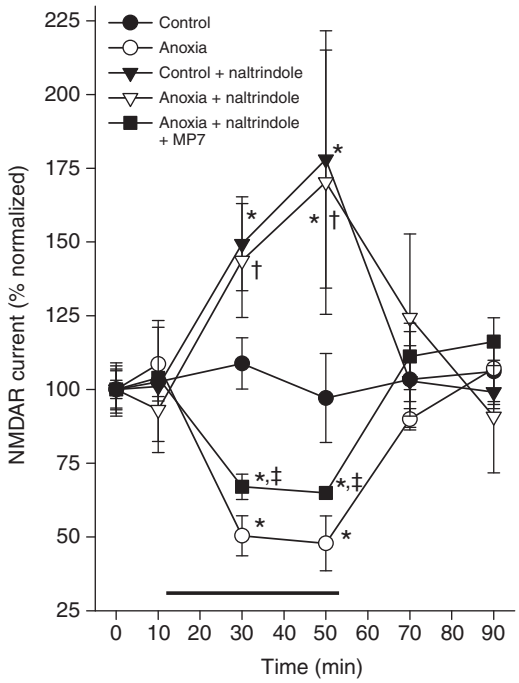

Fig. 3. Naltrindole potentiates normoxic NMDAR currents and reverses the anoxic decrease in NMDAR currents *via* G_i protein inhibition. Summary of changes in NMDAR currents during treatment. Data are expressed as means ± s.e.m. *Data significantly different from normoxic controls; †data significantly different from anoxic controls; ‡data significantly different from anoxic neurons treated with naltrindole. Significance was assessed at $P<0.05$. The bar indicates the duration of the treatment indicated in the key.

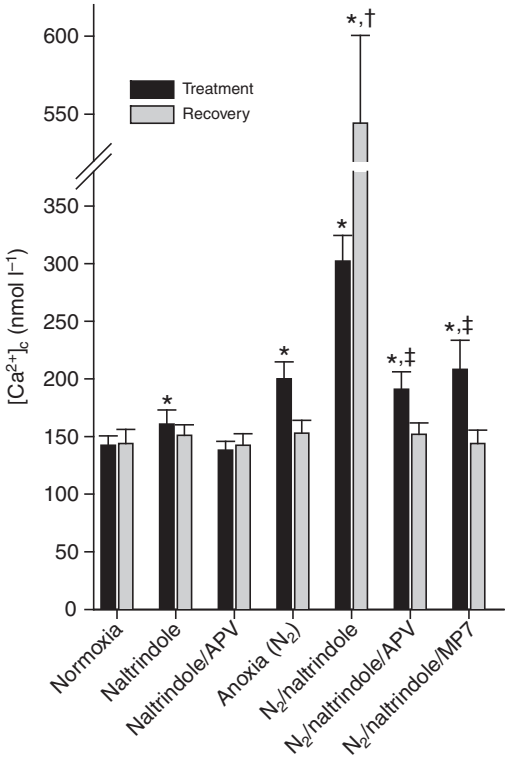

Fig. 4. Naltrindole induces severe NMDAR-mediated calcium influx. Summary of changes in $[Ca^{2+}]_c$ during normoxia and anoxia with and without the δ-opiod receptor (DOR) antagonist naltrindole alone or in the presence of APV or MP7. Data are expressed as means ± s.e.m. *Data significantly different from normoxic control; †data significantly different from anoxic control; ‡data significantly different from anoxia plus naltrindole treatment. Significance was assessed at $P<0.05$.

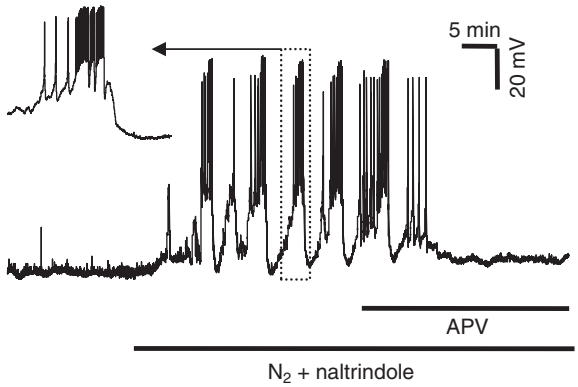

Fig. 5. NMDAR blockade abolishes excitatory events in anoxia plus naltrindole-treated neurons. Seizure-like events induced by perfusion of naltrindole during anoxia were abolished by co-perfusion of the NMDAR antagonist APV, preventing further anoxic depolarization and excitotoxic cell death.

anoxia alone and not that of anoxic plus naltrindole-treated neurons. Perhaps more importantly, in anoxic plus naltrindole-treated neurons already undergoing electrical hyper-excitability, APV perfusion both abolished the excitation and enhanced E_m depolarization (Fig. 5). APV also prevented $[Ca^{2+}]_c$ increases beyond changes observed in anoxic controls and $[Ca^{2+}]_c$ decreased significantly following reoxygenation (Fig. 1E; Fig. 4; N=4).

Blockade of DORs with naltrindole prevents DOR-mediated G_i cascades. MP7 is a direct stimulator of G_i proteins and perfusion of MP7 should relieve the naltrindole-mediated DOR blockade since it acts downstream of DORs and thus of naltrindole. Therefore, we co-perfused cortical sheets with MP7 and naltrindole during anoxia. MP7 prevented the naltrindole-mediated electrical hyper-excitability and severe depolarization was not observed (Fig. 1F; Fig. 2; N=6). Furthermore, the anoxic decrease in NMDAR activity was restored and NMDAR activity decreased (Fig. 1F; Fig. 3; N=6). Finally, large increases in $[Ca^{2+}]_c$ were not observed and cells behaved like control anoxic cells: $[Ca^{2+}]_c$ increased 46±3% with anoxia to 208±25 nmol l^{-1} and this change was reversed by reoxygenation (Fig. 1F; Fig. 4; N=5).

DISCUSSION

We have demonstrated here that DORs inhibit NMDAR-dependent Ca^{2+} influx during both normoxia and anoxia *via* a G_i-sensitive mechanism in turtle cortical neurons. DOR antagonism with naltrindole potentiated normoxic NMDAR currents and Ca^{2+} influx, suggesting that a basal level of DOR activity is critical to regulating NMDARs and $[Ca^{2+}]_c$. In anoxic neurons treated with naltrindole, terminal depolarization occurred in 31% of our experiments and a large depolarization of E_m was observed in all neurons that was not reversed by reperfusion of normoxic naltrindole-free ACSF. Concomitantly, anoxic elevations in $[Ca^{2+}]_c$ were greatly increased by naltrindole and were further increased following reoxygenation. This is contrary to control anoxia-alone experiments in which both the E_m depolarization and the increase in $[Ca^{2+}]_c$ were significantly smaller in magnitude and reversed following reoxygenation (Bickler, 1992; Bickler et al., 2000; Pamenter and Buck, 2008; Pamenter et al., 2008d). These deleterious events are likely to be dependent on NMDAR-mediated Ca^{2+} influx since naltrindole-mediated electrical hyper-excitability, severe prolonged depolarization and enhanced Ca^{2+} influx were abrogated in cortical slices pre-treated with the NMDAR antagonist APV. Furthermore, in cells exhibiting seizure-like events during anoxic naltrindole perfusion, APV application abolished hyper-excitability and E_m recovered following reoxygenation.

Turtle neurons employ numerous mechanisms to limit glutamatergic hyper-excitability during anoxia, including: depressed NMDAR activity and receptor abundance, reduced glutamate release, and decreased AMPA receptor activity (Bickler et al., 2000; Milton et al., 2002; Pamenter et al., 2008c). As a result, NMDAR-mediated damage due to toxic $[Ca^{2+}]_c$ accumulation and deleterious nitric oxide production are avoided in the anoxic turtle cortex (Bickler, 1992; Pamenter et al., 2008a). In previous studies examining turtle cortical NMDAR activity, neurons were voltage clamped and perfused with tetrodotoxin to prevent action potentials. As a result, the true importance of NMDAR regulation in anoxic neuronal survival could not be determined. In the present study, we directly correlate enhanced NMDAR activity with excessive $[Ca^{2+}]_c$ accumulation and terminal depolarization in cortical neurons, highlighting the critical role of NMDAR depression in the turtle's anoxia tolerance. Indeed turtle neurons exposed to naltrindole during anoxia responded in a fashion similar to mammalian neurons exposed to glutamate toxicity or ischemia. In such neurons, excessive NMDAR-mediated Ca^{2+} influx is observed along with severe depolarization (Coulter et al., 1992; Garthwaite and Garthwaite, 1986; Garthwaite et al., 1986). Furthermore, upon reoxygenation, mammalian E_m remains depolarized and, as in our experiments, these

effects can be prevented by perfusion of an NMDAR antagonist prior to the onset of severe depolarization (Limbrick et al., 2003).

The mechanism by which DORs and NMDARs interact is poorly understood, but as DORs are coupled to G_i proteins, the pathway probably involves G_i activation. There is some indirect evidence to support a role for G protein-mediated responses in the turtle's anoxia tolerance. Whole-brain cAMP concentration decreases significantly in the anoxic cortex, and since cAMP is directly mediated by G_i activity these data suggest anoxia-induced changes in G_i signaling occur in turtle brain (Pamenter et al., 2007). Furthermore, we have recently shown that the anoxic depression in turtle NMDAR activity is blocked by pertussis toxin, a G_i inhibitor (Pamenter et al., 2008b). Combined with our present report that the G_i activator MP7 depressed NMDAR currents and prevented extended depolarization and large accumulations of $[Ca^{2+}]_c$ following anoxic/naltrindole treatment, there is now mounting evidence that G_i signaling is critical to the regulation of NMDARs in the anoxic cortex. G proteins are well suited to act as anoxic messengers because they mediate numerous downstream mechanisms in neurons. Of particular interest are mitochondrial ATP-sensitive K^+ channels (mK_{ATP}), whose activity can be directly regulated by binding of G protein subunits to the sulfonylurea subunit of the mK_{ATP} channel (Wada et al., 2000). Recently we demonstrated that mK_{ATP} channels mediate the anoxic decrease in turtle NMDAR activity, such that activation of mK_{ATP} during anoxia partially uncouples mitochondria, decreasing the mitochondrial Ca^{2+}-buffering capacity and subsequently elevating $[Ca^{2+}]_c$, which regulates NMDARs *via* a phosphatase-dependent process (Bickler et al., 2000; Pamenter et al., 2008d; Shin et al., 2005). Although mK_{ATP} activation is critical to anoxic decreases in NMDAR activity, these channels are not oxygen sensitive and are thus unlikely to be the primary detector of low oxygen that initiates downstream NMDAR depression. Therefore, the pathway upstream of mK_{ATP} activation in turtle cortical NMDAR depression remains of interest.

Interestingly, just as anoxic turtle neurons treated with naltrindole resemble ischemic mammalian neurons, mammalian mechanisms of inducible neuroprotection may parallel the endogenous mechanism of NMDAR regulation in the anoxic turtle cortex. In mammals, DOR and mK_{ATP} activation are both critical to HPC-induced neuroprotection and agonists of either receptor are neuroprotective in a range of ischemia-tolerance models, including HPC (Chao et al., 2007a; Kis et al., 2003; Yoshida et al., 2004; Zhang et al., 2000). Since targeted blockade of either receptor prevents HPC-mediated neuroprotection in mammals and NMDAR depression in turtles, it is logical that the two receptors must function at separate but critical points in the same pathway. Indeed, there is evidence supporting this hypothesis: blockade of K_{ATP} channels abolishes DOR agonist-induced neuroprotection in ischemic mammalian heart and brain (Lim et al., 2004; Patel et al., 2002). The mechanism of DOR-/mK_{ATP}-induced neuroprotection in mammalian brain remains unresolved; however, regulation of NMDAR activity as occurs in the turtle is an attractive possibility as this regulation is critical to preventing ECD in mammalian neurons (Arundine and Tymianski, 2003; Pamenter et al., 2008d). This hypothesis is supported indirectly by the inverse relationship between NMDAR activity and DOR activation observed in mammalian brain whereby increases in DOR activity suppress NMDAR activity (Cao et al., 1997; Wang and Mokha, 1996). Therefore, the final mediator of neuroprotection in DOR-mediated HPC models may be a reduction in NMDAR current.

DOR protein expression in the turtle brain is high relative to that in rat brain tissue (Xia and Haddad, 2001), and this difference in receptor expression could partly explain the turtle's remarkable innate anoxia tolerance relative to the mammalian brain, despite their apparent reliance on similar neuroprotective mechanisms. Greater receptor expression may confer a higher sensitivity to opioid-mediated signaling, allowing the turtle brain to rapidly decrease NMDAR activity in response to decreasing environmental oxygen while long-term mechanisms of metabolic depression are being activated [e.g. removal of channels from neuronal membranes (Bickler et al., 2000; Perez-Pinzon et al., 1992)]. Conversely, mammalian brain requires HPC pre-treatment to upregulate DOR protein expression and 'prime' the brain against subsequent low-oxygen insults. Thus turtle brain is better able to respond quickly to low-oxygen insults than murine brain. Conversely, the role of DOR-mediated signaling in long-term anoxia tolerance in the turtle remains undetermined; however, NMDAR activity remains depressed during at least the first 6 weeks of anoxia in turtle brain and DOR-mediated pathways may regulate this depression (Bickler, 1998). DORs are activated endogenously by enkephalins and determination of anoxia-induced changes in turtle brain enkephalin concentration would be an informative next step in the elucidation of this pathway.

In addition to limiting glutamatergic excitability, the turtle brain exhibits a wide range of neuroprotective responses to anoxia. In general, mechanisms that are electrically inhibitory (e.g. GABAergic and adenosinergic) are elevated during anoxia, while excitatory mechanisms (e.g. glutamatergic) are depressed (Nilsson and Lutz, 1992; Nilsson et al., 1990). Experimentally, blocking NMDAR depression (with naltrindole), GABAergic activation (M.E.P. and L.T.B., unpublished observations) or adenosinergic signaling (Milton et al., 2007) all induce cell death in anoxic turtle neurons. Therefore, each of these mechanisms appears to be individually critical to anoxic survival. The existence of multiple neuroprotective responses to anoxia may be due to the activation of numerous oxygen-sensitive mechanisms that are initiated at sequentially lower P_{O_2} during a normoxic to anoxic transition. For example, adenosine receptors regulate NMDAR activity under hypoxic (5% O_2) but not anoxic (0% O_2) conditions (Pamenter et al., 2008b). The occurrence of overlapping oxygen-sensitive systems in the turtle brain is logical as this organism repeatedly experiences rapid changes in O_2 availability in its natural environment (Ultsch, 2006). Multiple oxygen sensors calibrated to detect changes in small ranges of P_{O_2} and subsequently initiate specific cellular responses to that P_{O_2} would be evolutionarily advantageous to this oxygen conformer and confer a greater degree of sensitivity in terms of cellular responses to changes in oxygen availability. Furthermore, the individual importance of each of these mechanisms suggests the metabolic state of the anoxic turtle brain is very tightly regulated and that any significant deviation from this state that increases electrical activity may quickly overcome the turtle neurons' ability to match its metabolic depression to decreased energy availability during anoxia. This may explain why naltrindole is neurotoxic in turtles during anoxia but not normoxia, despite potentiating NMDAR currents in both experiments. During normoxia, the Na^+/Ca^{2+} exchanger and the associated Na^+/K^+-ATPases that maintain the Na^+ gradient counter excessive NMDAR-mediated Ca^{2+} influx. However, during anoxia, turtle ATPase activity is decreased to match decreased ATP production (Buck and Hochachka, 1993; Buck and Pamenter, 2006; Hylland et al., 1997). Thus an increase of Na^+/Ca^{2+} exchanger activity to counter NMDAR-mediated Ca^{2+} influx would deplete the Na^+ gradient more rapidly than the ATPase can match in its reduced activity state, leading to rundown of E_m and deleterious $[Ca^{2+}]_c$ accumulation.

In conclusion, we have shown that blockade of DORs potentiates normoxic NMDAR currents and not only prevents but also reverses the anoxic decrease in NMDAR activity, leading to excitotoxic events, increased Ca^{2+} influx and terminal depolarization in a significant percentage of anoxic neurons. Our results offer a strong hypothesis to researchers examining mammalian models of HPC-mediated neuroprotection against ischemic insults. DOR activation is neuroprotective in mammalian models of stroke, and in both the turtle and mammals, DORs regulate NMDAR activity. As in turtle neurons, depression of NMDAR activity is critical to ischemic survival in mammalian neurons since blockade of NMDARs reduces $[Ca^{2+}]_c$ accumulation and prevents ECD following ischemic insult. However, direct blockade of NMDARs results in sedative or psychomimetic side-effects (Ikonomidou and Turski, 2002). DOR-mediated inhibition of NMDARs, as occurs in turtle cortex, may offer an indirect mechanism of NMDAR depression that is independent of direct pharmacological NMDAR blockade.

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