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Regulation of extracellular glutamate levels in the long-term anoxic turtle striatum: coordinated activity of glutamate transporters, adenosine, K^+_{ATP} channels and GABA

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Summary

Early in anoxia the mammalian brain experiences an uncontrolled release of glutamate, which combined with the failure of glutamate reuptake mechanisms, leads to massive neurotoxic increases in extracellular glutamate. By contrast, the anoxia tolerant turtle (*Trachemys scripta*) shows no increase in extracellular glutamate levels over many hours of anoxia. During the first hours of anoxia extracellular glutamate levels are maintained by a reduction in glutamate release (mainly due to the inhibition of neuronal vesicular glutamate release), combined with continued uptake by still active glutamate transporters. The early down-regulation in glutamate release is modulated by adenosine receptors and K^+_{ATP} channels, but is not affected by $GABA_A$ receptors. During long-term anoxia there is a further reduction in the rate of glutamate release, reaching 30% of normoxic control values at 5 h of anoxia. Adenosine and $GABA_A$ receptors but not K^+_{ATP} channels regulate this reduction in glutamate release. We conclude that the reduction in glutamate release during progressive anoxia is a dynamic process requiring continuous but changing synergistic activity of K^+_{ATP} channels, adenosine and $GABA_A$ receptors. The fact that there is a still active glutamate release and uptake in prolonged anoxia suggests that extracellular glutamate has a vital function in the deeply hypometabolic brain.

Introduction

In the mammalian brain extracellular glutamate levels are maintained at low (uM) concentrations by the coordinated balancing of glutamate release with glutamate re-uptake into the surrounding cells [1]. However, within minutes of oxygen deprivation, such as in anoxia and ischemia, the mammalian neuron suffers a depletion in ATP levels. Consequently there is a passive loss of transmembrane ionic gradients, depolarization and an uncontrolled increase in extracellular glutamate to neurotoxic levels. This massive rise in extracellular glutamate and the resulting cascade of cell death is central to ischemic and post-ischemic brain damage and is the focus of much research. Possible causes of the excessive rise in glutamate include (a) increased rate of release from vesicular and nonvesicular sources [2; 3], (b) a reversal of glutamate transporters [4] and (c) the failure of glutamate re-uptake mechanisms [2].

In contrast to the high vulnerability of the mammalian brain to hypoxia (as well as most other vertebrates), the brain of the freshwater

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turtle, *Trachemys scripta*, is able to withstand anoxia for days at room temperature [5, 6]. The turtle's first line of defense against anoxic brain failure is a drastic suppression of ATP demand and supply pathways, activated over a 1–2 h transition period [7, 8]. The second line of defense involves maintaining the cellular and circuit integrity of the deeply depressed brain (at an order of magnitude lower than normoxic levels) over hours to days of long-term anoxia [9].

The turtle brain avoids excitotoxic damage by maintaining low extracellular glutamate levels for at least 5 h of anoxia [10] and by reducing NMDA receptor activity [11]. We have recently shown that during the transition from the normoxic to the hypometabolic anoxic state, i.e. over the first 1.5 h of anoxia, glutamate release is decreased approximately 40% while glutamate re-uptake mechanisms remain active, with glial cells accounting for the majority of glutamate clearance [12]. During this period the inhibition of glutamate release is mediated by the activation of adenosine receptors and the opening of ATP sensitive potassium (K^+_{ATP}) channels [12].

This study examines the mechanisms that account for extracellular glutamate homeostasis during subsequent long-term anoxia. We aimed to determine the role of adenosine, K^+_{ATP} channels and γ-aminobutyric acid ($GABA_A$) receptors in the regulation of glutamate homeostasis. The role of K^+_{ATP} channels and GABA are particularly significant because there is evidence that the former is only effective during the initial anoxic period when ATP levels are temporarily depressed [13] and because extracellular GABA levels remain low during the transition into anoxia but gradually increase with extended anoxia [10], $GABA_A$ receptor activity is also up-regulated in the anoxic turtle brain [14].

In this investigation we found that glutamate homeostasis in the anoxic turtle striatum is the result of continued glutamate reuptake combined with both an inhibition of neuronal vesicular glutamate release and a reduction of glutamate release from nonvesicular sources. By blocking glutamate reuptake with PDC we show that $GABA_A$ receptor activation has a role in the inhibition of glutamate release during long-term anoxia, but not during early anoxia. K^+_{ATP} channel activation inhibits glutamate release only during early anoxia, but not during long-term anoxia whereas adenosine receptor activation inhibits glutamate release at all stages of anoxia.

Methods

All experiments were approved by the institutional animal care and use committee. Freshwater turtles (*Trachemys scripta*) were purchased from Lemberger in Oshkosh, WI. The general glutamate transporter blocker L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), the specific $GABA_A$ receptor agonist muscimol and the specific $GABA_A$ receptor antagonist bicuculline were purchased from Tocris Cookson (St. Louis, MO). The specific K^+_{ATP} channel blocker 2,3-butanedione monoxime (BDM) was purchased from Research Biochemicals International (Natick, MA). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Microdialysis methods

All experiments were performed at room temperature (25 °C) as previously described [12]. Turtles were anesthetized with AErrane (Isoflurane USP) by endotracheal intubation and ventilated with air using a small animal respirator (SAR-830 from CME, Inc.). Upon reaching a surgical plane, as indicated by lack of ocular reflex, a 1.5 cm area of skull was removed. A small incision in the dura mater was made above the cerebral hemisphere for microdialysis placement. A stereotaxic instrument was used to insert a CMA-12 microdialysis probe (3 mm membrane length, Bioanalytical Systems) into the striatum, 2.0 mm laterally of the midline, 2.0 mm caudal of the anterior cerebrum tip, and 4.0 mm ventral of the cerebrum surface [10]. An artificial cerebrospinal fluid (aCSF), (100 mM NaCl, 3.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, 2.0 mM glucose, pH 7.4) was perfused continuously through the microdialysis probe at a flow rate of 2.0 µl/min, via a CMA/100 microsyringe pump (Carnegi Medicine, Stockholm Sweden). Drugs, where indicated, were dissolved in aCSF and infused through the microdialysis probe. Elevated amino acid levels, caused by tissue damage during probe insertion, were allowed to return to baseline values, for 1 h, before sample collection began.

At the end of each experiment, methylene blue was perfused through the microdialysis probe and the brain removed, frozen and sectioned in order to check striatal probe placement.

Perfusate glutamate concentrations were determined by reverse-phase high performance liquid

chromatography (HPLC) with fluorescence detection (Shimadzu). In brief, 20 µl of the perfusate was mixed with 30 µl o-phthaldialdehyde (Sigma) for exactly one minute, after which 30 µl of the mixture was injected into the HPLC system. The inorganic mobile phase consisted of 90% deionized water, 10% methanol and 88 mM sodium acetate (pH 6.8) and the organic mobile phase consisted of 80% methanol and 20% deionized water. The mobile phases were pumped through an OPA Adsorbosphere C18 column (Alltech) at 1.3 ml/min. Glutamate concentrations were determined by comparison to known standards.

PDC, a general glutamate transport blocker, was either administered alone or co-administered with additional treatments, described below. PDC, which has high affinity for glutamate transporters and negligible affinity for glutamate receptors, reliably elevates extracellular glutamate levels in the mammalian brain without causing neurological damage [15]. The seemingly high concentrations of drugs typically used in microdialysis studies are necessary because of the incomplete permeability of the dialysis membrane and the torturous diffusion pathways through the tissue [16] such that only a fraction of the drug infused through a microdialysis probe actually reaches the tissue.

Glutamate release and re-uptake during prolonged periods of anoxia

Turtles were exposed to PDC in order to determine the rate of glutamate release during increasing periods of anoxia. After an initial 1 h recovery period, in which the turtles were ventilated with air, the gas mixture was switched to 100% nitrogen to induce anoxia. Glutamate baseline levels, the average of three consecutive 20 min samples, were collected after the turtle had been respired on nitrogen for 30 min, 2 or 4 h. The perfusate was then switched, via a liquid switch (CMA 110), to 2.5 mM PDC containing aCSF and three samples were collected for an additional hour. Baseline glutamate release during normoxia was determined after 1 h of air respiration and was followed by 1 h of PDC treatment.

Role of adenosine and K^+_{ATP} channels during long-term anoxia

The relative contributions of adenosine and K^+_{ATP} channels in reducing glutamate release during long-term anoxia (3–5 h) was determined using the general adenosine receptor antagonist, theophylline and the K^+_{ATP} channel blocker, BDM. Anoxic turtles were treated as described above (1 h baseline sampling followed by 1 h of drug treatment) except for the addition of 100 µM theophylline or 200 µM adenosine and/or 500 µM BDM to the 2.5 mM PDC containing aCSF.

Effects of $GABA_A$ receptor activation during normoxia

The ability of GABA to inhibit glutamate release was initially determined by perfusing the normoxic brain with the $GABA_A$ receptor agonist muscimol. Following an initial 1 h recovery period, three consecutive 20 min samples were collected in order to determine glutamate baseline level. This was followed by perfusing the brain with an aCSF in which 2.5 mM PDC had been dissolved or with an aCSF in which 100 µM muscimol and 2.5 mM PDC had been dissolved.

Role of GABA during early and long-term anoxia

Turtles were exposed to bicuculline, a $GABA_A$ receptor antagonist, to determine if increases in extracellular GABA levels are sufficient to inhibit glutamate release. After an initial 1 h recovery period, in which the turtles were ventilated with air, the breathing mixture was switched to 100% nitrogen to induce anoxia. Turtles were respired on nitrogen for 30 min or 4 h before glutamate baseline values, the average of three consecutive 20 min samples, were collected. The brain was then perfused with an aCSF containing 2.5 mM PDC or co-perfused with 2.5 mM PDC and 100 µM bicuculline. Three samples were collected for an additional hour.

Origin of glutamate inhibition

To determine which pool of glutamate, vesicular or cytoplasmic, is inhibited with the onset of anoxia, the brain was perfused with either a Ca^{2+} free aCSF or with tetrodotoxin (TTX), a voltage gated Na^+ channel blocker, in order to inhibit vesicular glutamate release. After recovery from microdialysis probe insertion, as described above, turtles were ventilated with either air or nitrogen for 30 min, after which baseline samples were collected

for 1 h. This was followed by an hour of sample collection in which the brain was perfused with either a Ca^{2+} free aCSF to which 200 μM EGTA [15] and 2.5 mM PDC was added or with an aCSF containing 10 μM TTX [17] and 2.5 mM PDC.

Glutamate baseline levels are the average of three samples collected for 1 h prior to experimental treatment. Data is expressed as percentage of baseline ± SE. SAS/JMP (Cary, NC) statistical package was used for data analysis. Statistical significance was determined nonparametrically using Kruskal–Wallis test for unequal variances, Tukey-Kramer post hoc analysis. In cases of equal variance, a one-way ANOVA/Student's t-test was used. $P < 0.05$ was considered to be statistically significant.

Results

Glutamate release and re-uptake continues during long-term anoxia

Basal levels of extracellular glutamate averaged 4.1 ± 0.83 μM in the turtle striatum; there was no significant difference between normoxic and anoxic baseline levels. Blocking glutamate re-uptake with PDC, in the normoxic turtle brain, resulted in an increase in extracellular glutamate levels of 510 ± 19% (Figure 1). The increase in extracellular glutamate accumulation was of a smaller magnitude under anoxic conditions. Exposing the turtle to 1.5 or 3 h of anoxia, while inhibiting glutamate re-uptake resulted in increases of 310 ± 29% and 320 ± 20%, respectively, in extracellular glutamate levels. Exposing the turtle to 5 h of anoxia while inhibiting reuptake resulted in a modest increase in extracellular glutamate levels to 200 ± 25% of basal. These results indicate that glutamate release is progressively inhibited over 5 h of anoxia and demonstrates that glutamate transporters must remain active throughout long-term (5 h) anoxia.

Vesicular glutamate release is inhibited during anoxia

In the normoxic turtle brain, the blocking of action-potential-dependent vesicular glutamate release with TTX while preventing glutamate reuptake with PDC resulted in a modest increase in extracellular glutamate accumulation (280 ± 32%) compared to non-TTX controls (Figure 2). Since vesicular glutamate release could also occur through direct depolarization of the axon terminal, the brain was perfused with a Ca^{2+} free aCSF. Blocking glutamate re-uptake in the absence of Ca^{2+} produced an almost identical reduction in the PDC induced increase in extracellular glutamate accumulation (280 ± 33%) as was found with TTX treatment. The reduction in extracellular glutamate levels caused by Ca^{2+} omission or TTX in normoxia is similar to that seen with the onset of anoxia (39%). Inhibiting vesicular glutamate release, with TTX or Ca^{2+} omission in early anoxia (1.5 h) did not significantly alter PDC induced extracellular glutamate accumulation (270 ± 44%, Ca^{2+} free and 260 ± 58%, TTX) when compared to the anoxic/PDC group (Figure 2). These results suggest that vesicular glutamate release is inhibited during early (0–1.5 h) anoxia.

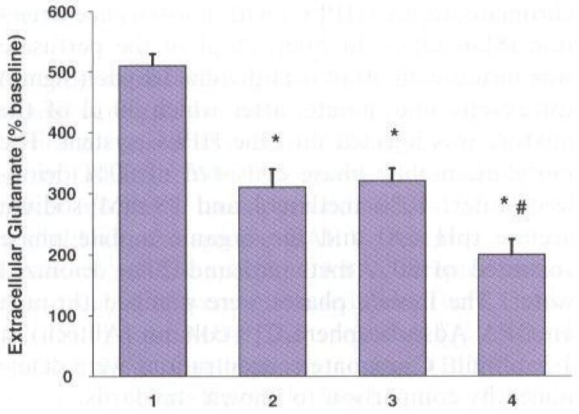

Figure 1. Effects of anoxia on glutamate release in the turtle striatum. Perfusing the normoxic or anoxic turtle striatum with PDC, a general glutamate transport blocker, increased extracellular glutamate levels at each of the time points investigated [normoxia (1), 1.5 h anoxia (2), 3 h anoxia (3), 5 h anoxia (4)]. This indicates that glutamate transporters continue to function during anoxia; a requirement since glutamate continues to be released. Interestingly, there is a progressive reduction in glutamate release during anoxia. There was no significant difference between normoxic and anoxic baseline levels (normoxic 4.4 ± 0.52; 1.5 h anoxia 3.8 ± 0.36; 3 h anoxia 4.3 ± 0.45; 5 h anoxia 3.9 ± 0.42 μM). Values are mean ± SE, $n = 5$ animals/group. *significantly different ($p < 0.05$) from normoxia and # is significantly different ($p < 0.05$) from 1.5 and 3 h anoxia.

Synergistic inhibition of glutamate release during 3 h of anoxia

After 3 h of anoxia the turtle brain was perfused with either BDM, a K^{+}_{ATP} channel blocker or the

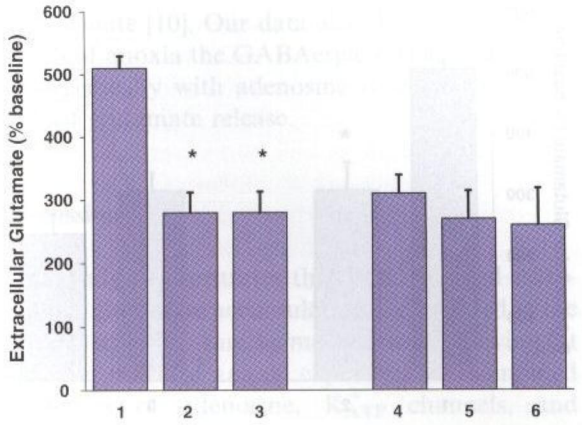

Figure 2. Effects of anoxia on neuronal vesicular glutamate release. Coperfusion of 10 μM tetrodotoxin (TTX) or a $-Ca^{2+}$ aCSF with the glutamate uptake inhibitor PDC reduced the rate of glutamate release in the normoxic but not the 1.5 h anoxic turtle brain. [normoxia (1), normoxia +TTX (2), normoxia $-Ca^{2+}$ (3), 1.5 h anoxia (4), 1.5 h anoxia + TTX (5), 1.5 h anoxia $-Ca^{2+}$ (6)] There was no significant difference between normoxic, anoxic, or drug treated baseline levels (normoxic +TTX 4.4 ± 0.52; air $-Ca^{2+}$ 4.0 ± 0.33; 1.5 h anoxia +TTX 3.6 ± 0.45; anoxia $-Ca^{2+}$ 4.5 ± 0.85 μM). Values are mean ± SE, n = 5 animals/group. * significantly different ($p < 0.05$) from normoxic controls (PDC only) animals.

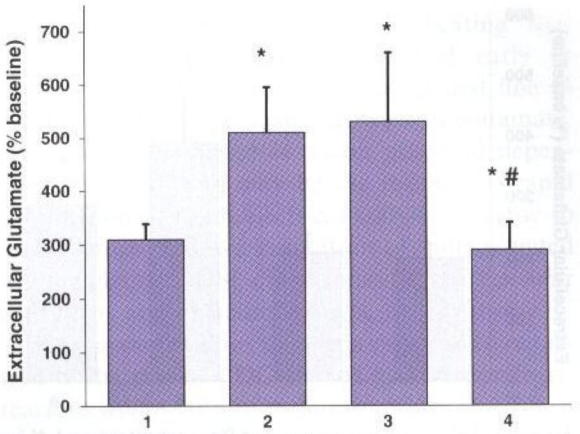

Figure 3. Effects of adenosine and K^+_{ATP} channels on glutamate release during 3 h of anoxia. Blocking either adenosine receptors (3) with 100 μM theophylline (Theo) (+PDC) or K^+_{ATP} channels (2) with 500 μM BDM (+ PDC) increased the rate of glutamate release to levels significantly different from 3 h anoxic (1) control turtles but not significantly different from normoxic control turtles. Addition of 200 μM adenosine (4) is able to inhibit glutamate release caused by BDM (+PDC) alone. There was no significant difference between anoxic or drug treated baseline levels (anoxia +Theo 4.0 ± 0.68; anoxia +BDM 3.7 ± 0.43; anoxia +BDM+Ado 4.5 ± 0.36 μM) Values are mean ± SE, n = 5 animals/group. * significantly different ($p < 0.05$) from N2 and # is significantly different ($p < 0.05$) from N2/PDC/BDM.

general adenosine receptor antagonist, theophylline in order to investigate the activity of adenosine and K^+_{ATP} channels on glutamate release. Coperfusing the brain with PDC and either BDM or theophylline produced similar increases in extracellular glutamate level (510 ± 85% and 530 ± 130% respectively, Figure 3). These values are significantly greater than perfusing the anoxic brain with PDC alone but not significantly different from normoxic/PDC animals. Simultaneously blocking K^+_{ATP} channels and adenosine receptors (in the presence of PDC) did not further increase extracellular glutamate accumulation compared to BDM (+PDC) or theophylline (+PDC) alone (data not shown). These results indicate that at 3 h of anoxia, both adenosine and K^+_{ATP} channels contribute to the reduction in glutamate release.

We have previously shown that perfusing the normoxic turtle brain with adenosine (while blocking glutamate reuptake with PDC) resulted in a reduction in glutamate release to levels seen during anoxia [12]. To test if adenosine receptors are only partially activated during 3 h of anoxia, the brain was perfused with 200 μM adenosine while simultaneously closing K^+_{ATP} channels with BDM. Coperfusing the brain with adenosine and BDM in the presence of PDC resulted in a smaller increase in extracellular glutamate accumulation (290 ± 51% Figure 3). This increase was significantly less than that obtained by perfusing the brain with BDM (+PDC) alone and was not significantly different from that of the 3 h anoxic/PDC group. These results indicate that adenosine receptor activation is only partially expressed at 3 h of anoxia and that regulation of glutamate release requires the synergistic activity of both adenosine and K^+_{ATP} pathways at this period.

Adenosine receptor activation inhibits glutamate release during long-term anoxia

The activity of adenosine receptors and K^+_{ATP} channels on glutamate release was investigated during long-term (5 h) anoxia. Perfusing the anoxic turtle brain with theophylline/PDC increased extracellular glutamate levels 390 ± 75%, which was signficantly higher than that of anoxic/PDC control animals (Figure 4). Perfusing the brain with BDM in the presence of PDC did not significantly increase extracellular glutamate levels when compared to

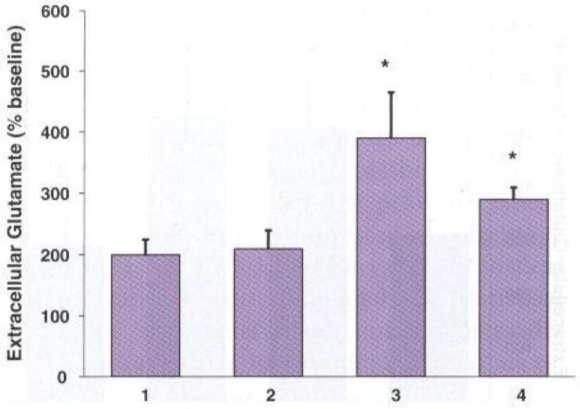

Figure 4. Effects of adenosine, $GABA_A$ receptors and K^+_{ATP} channels on glutamate release during prolonged periods of anoxia. Coperfusing the anoxic turtle brain with theophylline (Theo) + PDC (3) or bicuculline (Bic) + PDC (4) resulted in a significant increase in extracellular glutamate levels when compared to 5 h anoxic (PDC) controls (1). A significant increase in extracellular glutamate was not seen when the brain was perfused with BDM (+ PDC) (2). There was no significant difference between anoxic or drug treated baseline levels (anoxia + Theo 3.9 ± 0.79; anoxia + BDM 4.2 ± 0.82; anoxia + Bic 3.6 ± 0.62 μM). Values are mean ± SE, n = 5 animals/group. * significantly different ($p < 0.05$) from N2/PDC turtles.

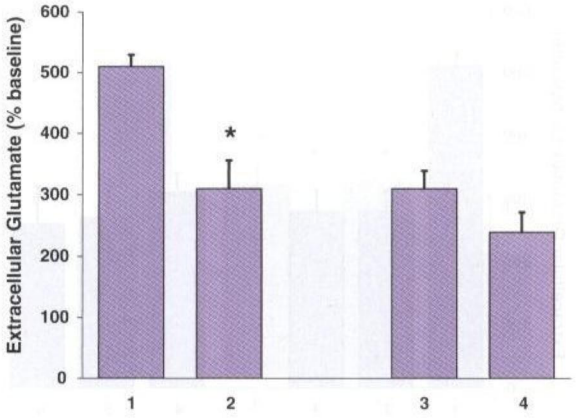

Figure 5. $GABA_A$ receptors do not modulate glutamate release during early anoxia. Coperfusing the normoxic brain with muscimol (Mus) in the presence of PDC (2) decreased the rate of glutamate release when compared to normoxic (+PDC) control turtles (1). However, no significant changes in PDC-evoked extracellular glutamate accumulation was found in the 1.5 h anoxic turtle brain when $GABA_A$ receptors were blocked with 100 μM bicuculline (Bic) in the presence of PDC (4). 1.5 h anoxic control turtles (+ PDC) (3). There was no significant difference between baseline levels (normoxia + Mus 4.6 ± 0.53; 1.5 h anoxia + Bic 4.0 ± 0.32 μM). Values are mean ± SE, n = 5 animals/group. * significantly different ($p < 0.05$).

anoxic/PDC animals. Co-perfusing the brain with theophylline and BDM (in the presense of PDC) increased extracellular glutamate to levels that did not differ from the theophylline/PDC group (data not shown). These results indicate that during prolonged anoxia adenosine continues to contribute to the regulation of glutamate release, while K^+_{ATP} channels are no longer functionally involved in this process. The increase in PDC-evoked glutamate release produced by inhibition of adenosine receptors at 5 h anoxia (1.8 fold over 5 h anoxia control) is very similar to that produced at 1.5 h (1.6 fold higher than the 1.5 h anoxia control) and 3 h (1.7 fold higher than the 3 h anoxia control), suggesting that the same pool of glutamate may be inhibited at each time point.

GABA receptor activation inhibits glutamate release at 5 h of anoxia but not at 1.5 h anoxia

Perfusing the normoxic brain with the $GABA_A$ receptor activator muscimol (+ PDC) resulted in a significantly smaller increase in extracellular glutamate accumulation (310 ± 46%) compared to the normoxic control (Figure 5). This observation indicates clearly that the $GABA_A$ receptor is ready to inhibit glutamate release even during normoxia.

To determine if GABA inhibits glutamate release during anoxia, the turtle was exposed to anoxia while $GABA_A$ receptors were blocked with bicuculline. During early anoxia (1.5 hr), co-perfusing the brain with 100 μM bicuculline + PDC increased extracellular glutamate levels by 240 ± 32%, to a level that was not significantly different from the 1.5 h anoxia (+PDC) group (310 ± 29%) (Figure 5). However, at 5 h of anoxia, bicuculline +PDC infusion produced a greater increase in extracellular glutamate levels (290 ± 19%) compared to the 5 h anoxia /PDC control group (200 ± 25%) (Figure 4). These results indicate that during the transition from the normoxic state to the metabolically depressed anoxic state $GABA_A$ receptors are not functionally involved in suppressing glutamate release. By contrast, it is clear from our data that in prolonged anoxia the GABAergic system contributes to glutamate inhibition through $GABA_A$ receptor activation. This involvement of the GABAergic system in regulating neurotransmission during prolonged anoxia is consistent with previous studies demonstrating that extracellular GABA levels remain low during the transition into anoxia but gradually increase throughout the established

anoxic state [10]. Our data also demonstrates that at 5 h of anoxia the GABAergic system is working synergistically with adenosine to elicit an inhibition of glutamate release.

Discussion

This study demonstrates that PDC induced extracellular glutamate accumulation is decreased in the anoxic turtle striatum by mechanisms involving (at differing times of anoxic exposure) the combined influences of adenosine, K^+_{ATP} channels, and $GABA_A$ receptors.

Our data indicates that in the turtle brain, extracellular glutamate homeostasis is maintained during prolonged anoxia by a progressive decrease in the rate of glutamate release and the continued activity of glutamate transporters (Figure 6). By contrast extracellular glutamate levels rapidly increase to destructive levels in the energy deprived mammalian brain, a result of an uncontrolled release of glutamate from vesicular and nonvesicular sources combined with a failure in glutamate uptake mechanisms [3, 17].

Inhibiting vesicular glutamate release in the normoxic brain with TTX or $-Ca^{2+}$ indicates that in the normoxic turtle brain about 50% of the PDC-evoked glutamate release is vesicular in origin, a value similar to that found for mammals [15]. By contrast, inhibiting vesicular glutamate release during anoxia did not affect the rate of PDC-evoked glutamate release indicating that vesicular glutamate release is halted early in anoxia. This inhibition is probably a first line of defense against neurotoxic increases in glutamate. Since vesicular release is action potential dependent, the inhibition may be the result of a rapid suppression in brain electrical activity [18] due to such factors as down regulation of voltage gated sodium channels [19], direct inhibition of vesicular exocytosis and/or neuronal hyperpolarization.

As anoxia develops there is a progressive reduction in the rate of PDC-evoked glutamate release, reaching about 60% of normoxic values at 1.5 h of anoxia and 30% at 5 h. This reduction is most likely due to a developing suppression of the non-vesicular glutamate release pathways, but the specific routes are not yet known. In mammalian organotypic hippocampal slices a continuous release of nonvesicular glutamate has been shown to occur under normal conditions [20]. However, the mechanism of release was unclear although it was shown not to involve cell swelling, transmembrane diffusion, or transporter reversal [20]. Interestingly there is a corresponding progressive down regulation of turtle cortical NMDA receptors during anoxia [11] which is similar to our current observations of a biphasic inhibition of glutamate release.

It appears that the signaling/ control mechanisms of PDC induced extracellular glutamate accumulation differ depending on the length of anoxia exposure. In the initial transition from normoxia to anoxia, when there is a substantial

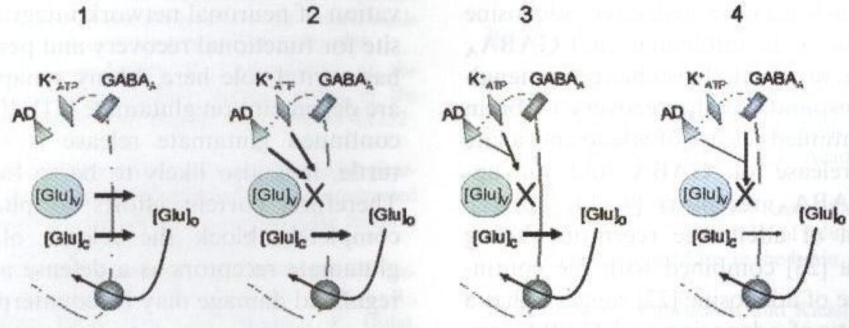

Figure 6. Proposed model of glutamate regulation in the anoxic turtle striatum. Glutamate homeostasis in the anoxic turtle striatum is the result of continued glutamate reuptake combined with a biphasic inhibition of glutamate release. Regulation of extracellular glutamate levels during normoxia is shown in 1. Inhibition of vesicular glutamate release ([Glu]v) occurs with the onset of anoxia and is regulated by the coordinated but changing activity of adenosine receptors (AD) and K^+_{ATP} channels during early anoxia [1.5 h (2) and 3 h (3)] and by adenosine and $GABA_A$ receptors during long-term anoxia [5 h (4)]. As the anoxic state develops (5 h) glutamate release is further inhibited by a reduction in cytoplasmic glutamate ([Glu]c) release. The enhanced release of GABA and the cyclic release of adenosine combined with the up-regulation in $GABA_A$ receptors and the stabilization of adenosine receptors suggests that the inhibitory effects of adenosine and GABA on glutamate release may persist throughout long-term anoxia.

down regulation in ATP consuming pathways, both adenosine receptors and K^+_{ATP} channels are initiators of glutamate inhibition [12]. During this period there is a temporary fall in ATP levels [7, 8] which results in the activation of K^+_{ATP} channels, and the release of adenosine [21, 22], which results in the activation of adenosine receptors. The activation of each pathway appears sufficient to produce the full inhibitory effect on glutamate release since it is only when both systems are antagonized that the anoxia induced decrease in glutamate release is prevented [12]. This indicates a redundancy or back up in control mechanisms during the critical initial periods of anoxia when ATP levels are low. The inhibitory effects of adenosine and K^+_{ATP} channels on PDC-evoked glutamate release, continues during later anoxic exposure when ATP levels are recovering. However the level of activation of each system is reduced such that inhibition of one pathway is sufficient to ameliorate the reduction in PDC-evoked glutamate release. This suggestion is supported by our observation that low extracellular glutamate levels were maintained upon blocking K^+_{ATP} channels, if the level of adenosine receptor activation was enhanced. Likewise, a similar progressive reduction in the effectiveness of K^+_{ATP} channels to down-regulate ion channels has been reported in the anoxic turtle cortex [13]. This reduction in pathway activation requires adenosine and K^+_{ATP} channels to work synergistically during this time period to inhibit glutamate release. During this period $GABA_A$ receptors do not influence glutamate release.

In the later long-term hypometabolic anoxic state, K^+_{ATP} channels become ineffective, adenosine receptors continue to be influential, and $GABA_A$ receptors become involved. This change in signaling activity corresponds to the recovery of brain ATP levels, a continued release of adenosine, a late and sustained release of GABA and an up-regulation in $GABA_A$ receptors [7, 14, 21, 22]. The stabilization of adenosine receptors during long-term anoxia [23] combined with the continued, cyclic release of adenosine [22] suggests that a synergistic activity of adenosine and GABA may persist throughout long-term anoxia.

The neuromodulatory effects of adenosine, K^+_{ATP} channels and $GABA_A$ receptors appear to primarily involve the inhibition of vesicular glutamate release. This is based upon (a) activation of either adenosine receptors, K^+_{ATP} channels or $GABA_A$ receptors, in the normoxic turtle brain, is able to inhibit glutamate release to levels identical to that seen with the onset of anoxia and (b) inhibiting adenosine receptors and K^+_{ATP} channels, in the anoxic turtle brain, is able to increase the rate of glutamate release to normoxic levels. In the mammalian brain each of these pathways have been shown to regulate glutamate release [24–26].

The data presented here suggests that the turtle brain moves from an excitatory to an inhibitory tone with the onset of anoxia using processes similar to those elicited by preconditioning in the mammalian brain, where a sublethal ischemic insult provides temporary protection from otherwise lethal exposures. The neuroprotective effects of preconditioning are associated with a reduction in glutamate release, enhancement of GABA release, up-regulation of $GABA_A$ receptors and a down-regulation of NMDA and AMPA receptors [27, 28]. Adenosine and K^+_{ATP} channels are also associated with ischemic preconditioning and may be involved in the initial events of ischemia tolerance [29]. The turtle appears to be constitutively "preconditioned" with inhibitory tones being initiated by adenosine and K^+_{ATP} channels and further strengthened through enhanced GABA release and up-regulation of $GABA_A$ receptors.

Since the uptake of glutamate is energetically expensive, estimated at 1.5 ATP per glutamate anion [30], and the turtle's basic strategy to survive anoxia is to minimize ATP expenditures, the continued transporter activity suggests that the continued release and uptake of glutamate has an important function in anoxia survival. The preservation of neuronal network integrity is a prerequisite for functional recovery and perhaps glutamate has a vital role here. Many synaptic connections are dependant on glutamate [31]. If some degree of continued glutamate release is essential to the turtle, it is also likely to be so for the mammal. Therefore, current efforts to pharmacologically completely block the release of glutamate or glutamate receptors as a defense against ischemia regulated damage may be counterproductive.

Acknowledgements

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References

1. Bradford H.F., Young A.M.J. and Crowder J.M., Continuous glutamate leakage from brain cells is balanced by compensatory high-affinity reuptake transporter. Neurosci. Lett. 81: 296–302, 1987.
2. Jabaudon D., Scanziani M., Gahwiler B.H. and Gerber U., Acute decrease in net glutamate uptake during energy deprivation. Proc. Natl. Acad. Sci. 97: 5610–5615, 2000.
3. Pocock J.M. and Nicholls D.G, Exocytotic and nonexocytotic modes of glutamate release from cultured cerebellar granule cells during chemical ischemia. J. Neurochem. 70(2) 806–813, 1998.
4. Rossi D.J., Oshima T. and Atwell D, Glutamate release in severe brain ischemia is mainly by reversed uptake. Nature 403: 316–322, 2000.
5. Lutz P.L., Nilsson G.E. and Prentice H., The Brain Without Oxygen: Causes Of Failure Molecular and Physiological Mechanisms for Survival. 3rd edn. Kluwer Press, 2003.
6. Bickler P.E, Donohoe P.H. and Buck L.T., Molecular adaptations for survival during anoxia: lessons from lower vertebrates. Neuroscientist 8(3) 234–242, 2002.
7. Lutz P.L, McMahon P., Rosenthal M. and Sick T., Relationship between aerobic and anaerobic energy production in turtle brain in situ. Am. J. Physiol. 247: R740–R744, 1984.
8. Kelly D.A. and Storey K.B., Organ specific control of glycolysis in anoxic turtles. Am. J. Physiol. 255(1088): R774–R778, 1988.
9. Hochachka P.W. and Lutz P.L, Mechanism, origin and evolution of anoxia tolerance in animals. Comp. Biochem. Physiol. B Biochem. Mol. Bio. 130(4) 435–459, 2001.
10. Nilsson G.E. and Lutz P.L, Release of inhibitory neurotransmitters in response to anoxia in turtle brain. Am. J. Physiol. 261(30) R32–R37, 1991.
11. Bickler P.E, Donohoe P.H. and Buck L.T, Hypoxia-induced silencing of NMDA receptors in turtle neuron. J. Neurosci. 20(10) 3522–3528, 2000.
12. Milton S.L., Thompson J.W. and Lutz P.L, Mechanisms for maintaining extracellular glutamate levels in the anoxic turtle striatum. Am. J .Physiol. 282: R1317–R1323, 2002.
13. Pek-Scott M. and Lutz P.L, ATP-sensitive K+ channel activation provides transient protection to the anoxic turtle brain. Am. Physiol. Soc. 275(44) R2023–R2027, 1998.
14. Lutz P.L. and Leone-Kabler S, Upregulation of the GABAA/benzodiazepine receptor during anoxia in the freshwater turtle brain. Am. Physiol. Soc. 268(37) R1332–R1335, 1995.
15. Rawls S.M. and McGinty J.F, L-trans-pyrrolidine-2,4-dicarboxylic acid – evoked striatal glutamate levels are attenuated by calcium reduction, tetrodotoxin and glutamate receptor blockade. J. Neurochem. 68: 1553–1563, 1997.
16. Shippenberg T.S. and Thompson A.C., Overview of microdialysis. In: Crawley J.N., Gerfen C.R., McKay R., Rogawski M.A., Sibley D.R. and Skolnick P. (Eds), Current Protocols in Neuroscience. Wiley, New York, 1997, pp. 7.1.1–7.1.22.
17. Dawson L.A., Djali S., Gonzales C., Vinegra M.A and Zaleska M.M., Characterization of transient focal ischemia-induced increases in extracellular glutamate and aspartate in spontaneously hypertensive rats. Brain. Res. Bull. 53(6) 767–776, 2000.
18. Fernandes J.A., Lutz P.L., Tannenbaum A., Todorov T., Liebovitch .L and Vertes R., Electroencephalogram activity in the anoxic turtle brain. Am. J. Physiol. R273: R911–R919, 1997.
19. Perez-Pinzon M.A., Rosenthal M., Sick T.J., Lutz P.L., Pablo J. and Mash D., Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain. Am. J. Physiol. 262(4 pt 2) R712–R715, 1992.
20. Jabaudon D., Shimamoto K., Yasuda-Kamatani Y., Scanziani M., Gahwiler B.H. and Gerber U., Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. Proc. Natl. Acad. Sci. 96: 8733–8738, 1999.
21. Nilsson G.E. and Lutz P.L., Adenosine release in the anoxic turtle brain: a possible mechanism for anoxic survival. J. Exp. Biol. 162: 345–351, 1992.
22. Lutz P.L. and Kabler S., Release of adenosine and ATP in the brain of the freshwater turtle (Trachemys scripta) during long-term anoxia. Brain. Res. 796: 281–281, 1997.
23. Lutz P.L. and Manuel L., Maintenance of adenosine A1 function during long-term anoxia in the turtle brain. Am. Physiol. Soc. 276(45) R633–R636, 1999.
24. Goda H., Ooboshi H., Nakane H., Ibayashi S., Sadoshima S. and Fujishima M., Modulation of ischemia-evoked release of excitatory and inhibitory amino acids by adenosine A1 receptor agonist. Euro. J. Pharmacol. 35: 149–155, 1998.
25. Nelson R.M., Green A.R., Lambert D.G. and Hainswoth A.H., On the regulation of ischemia-induced glutamate efflux from rat cortex by GABA; in vitro studies with GABA, clomethiazole and pentobarbitone. British J. Pharm. 130: 1124–1130, 2000.
26. Matsumoto N., Kumamoto E., Furue H. and Yoshimura M., GABA mediated inhibition of glutamate release during ischemia in substantia gelatinosa of the adult rat. J Neurophysiol. 89: 257–264, 2003.
27. Grabb M.C., Lobner D., Turetsky D.M. and Choi D.W., Preconditioned resistance to oxygen-glucose deprivation-induced cortical neuronal death: alterations in vesicular GABA and glutamate release. Neuroscience 115(1) 173–183, 2002.
28. Swanson R.A. and Duan S., Regulation of glutamate transporter function. Neuroscience 5: 280–282, 1999.
29. Heurteaux C., Lauritzen I., Widmann C. and Lazdunski M., Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. Proc. Natl. Acad. Sci. 92: 4666–4670, 1995.
30. Takahashi M., Billups B., Rossi , Sarantis D., Hamann M. and Atwell D., The role of glutamate transporters in glutamate homeostasis in the brain. J. Exp. Biol. 200: 401–409, 1997.
31. Sommer C., Fahrner A. and Kiessling M, [^{3}H] Muscimol binding to γ-aminobutyric acid A receptors is upregulated in CA1 neurons of the gerbil hippocampus in the ischemia-tolerant state. Stroke 33: 1698–1705, 2002.