Survival of neurons in the brain of the extremely anoxia-tolerant Western painted turtle (Chrysemys picta) is associated with depression in the activity of key ion channels that preserve critical ion gradients (2, 5, 17). These changes are part of a suite of adaptations that are thought to coordinately decrease ion pumping and energy expenditure in hypoxia-tolerant cells (10, 11). An example of an important ion channel undergoing inactivation during anoxia in the turtle brain during anoxia is the N-methyl-D-aspartate (NMDA) receptor, which decreases in activity by 30–40% (4, 5, 17). The NMDA receptor mediates fast excitatory neurotransmission and, during hypoxic ischemic insults in the mammalian brain, contributes to uncontrolled Ca2+ influx and Ca2+-mediated cell injury (7). In turtles, alterations in NMDA receptor activity during the weeks to months of anoxia experienced during submergence dormancy could be important for survival, but long-term changes in receptor properties have not been studied.

One of the key factors thought to be important for the downregulation of ion channel function during anoxia in turtles is the accumulation of adenosine in the brain extracellular space (14, 16, 17). Adenosine has been shown to reduce the activity of potassium channels (17) and to partially explain the reduction in NMDA receptor ion channel activity as well (4, 5). However, adenosine levels increase over a 2-h period after the onset of anoxia and thereafter decrease toward baseline (16), leaving the role of adenosine in long-term receptor inactivation, such as that presumed to occur during prolonged dormancy, unclear.

Dramatic changes in the ionic composition of cerebrospinal fluid (CSF) of turtles during anoxic dormancy have important implications for the regulation of ion channels. The NMDA receptor, which is permeable to Ca2+ and Na+, is modulated by several extracellular ions whose concentrations change profoundly during prolonged anoxia. The NMDA receptor is inhibited by elevated magnesium and acidity (1, 22), whereas increased CSF Ca2+ levels will increase Ca2+ influx via the receptor channel (1). During 10 days of anoxia at 10°C, CSF Ca2+ concentration increases almost sixfold and Mg2+ concentration increases fivefold. pH decreases from 7.6 to 7.2, which should decrease NMDA receptor activity by 50% (22). We hypothesized that the increase in Mg2+ and acidity that occurs in CSF during anoxia depresses NMDA receptor activity sufficiently to compensate for the increased CSF Ca2+ concentration. To test this hypothesis, we measured NMDA receptor activity in turtle cerebrocortex during 6 wk of anoxia.

**METHODS**

With the approval of the University of California, San Francisco, Committee on Animal Research, 1–1.5 kg adult Western painted turtles (Chrysemys picta bellii) were obtained from commercial suppliers and housed under 12:12-h light-dark photoperiods at 25°C. Studies were conducted during the fall and winter months when turtles are normally dormant. Turtles were placed in 5- or 10-gallon aquariums in a cold room at 2–4°C for several days to allow thermal acclimatization. Nitrogen gas was then continuously bubbled through the water. The aquariums were then sealed for periods between 2 h and 6 wk. A small gas head space at the top of the aquarium was allowed to exist for 1–2 days. Turtles became inactive and minimally responsive during this time. Samples of water showed PO2 was <5 mmHg. Two turtles not allowed a period of acclimation with a gas head space above the aquarium water died, but 18 other animals survived up to 6 wk without apparent injury.
To study the activity of cortical NMDA receptors, cortical sheets, containing a superficial layer of cortical neurons with abundant NMDA receptors (23), were incubated in turtle artificial CSF (aCSF) containing the Ca$^{2+}$ indicator turta 2. The methods for dissecting, loading slices with turta 2, and measuring intracellular Ca$^{2+}$ changes are exactly as described in Buck and Bickler (4). Cortical sheets from anoxic turtles were dissected and turta 2-loaded in a nitrogen atmosphere (Atmos bag, Fisher Scientific). Four to six sheets were obtained from each animal. During turta loading, slices were continuously bubbled with 95% N2 and 3% CO2. NMDA receptor-mediated changes in intracellular Ca$^{2+}$ were measured during application of NMDA (final concentration 100 µM) to cortical sheets in a fluorometer cuvette. To prevent the exogenous NMDA from triggering action potentials and releasing neurotransmitters, sheets were incubated in 1 µM tetrodotoxin, 0.1 µM ω-conotoxin, and 0.5 µM agatoxin GIVA. With these sodium and Ca$^{2+}$ channel blockers present, we assumed that application of NMDA triggered Ca$^{2+}$ changes almost exclusively due to Ca$^{2+}$ influx through the activated NMDA receptor complex (9).

The activity of the NMDA receptor during normoxia and 6 wk of anoxia was compared using two benchmarks: the response of the receptor in normal Ca$^{2+}$ and in aCSF with the higher Ca$^{2+}$ found in anoxic animals. This was done to follow the changing activity of the NMDA receptor in reference to a normoxic control and to discover if the response of the receptor to high Ca$^{2+}$ changes during long-term anoxia. Basal intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]) and Ca$^{2+}$ changes after NMDA application were measured in one of two types of aCSF. The first was standard aCSF containing the following ions present in the CSF of normoxic turtles (in mM): 129 Na$,^+$, 91 Cl$^-$, 1.9 K$,^+$, 1.3 Ca$^{2+}$, 1.2 Mg$^{2+}$, 2.9 lactate, 10 glucose, and 41 HCO$_3$$. The second was aCSF containing ions found in the CSF of turtles after 10 days of anoxic dormancy (8): 131 Na$,^+$, 62 Cl$^-$, 4.0 K$,^+$, 8.4 Ca$^{2+}$, 5.1 Mg$^{2+}$, 69 lactate, 10 glucose, and 31 HCO$_3$. Slices were randomly assigned for treatment with one of these media and were studied only once.

**Results**

Eighteen of twenty turtles survived up to 6 wk of anoxia without apparent injury. Although the blood of these turtles was black in color at the time of brain dissection, the heart of all animals was beating at the time of death. The brain tissue of turtles after 1 or more weeks of anoxia appeared softer and more edematous than normoxic controls. The brain tissues of turtles recovered in air (at room temperature) for 1 day after 6 wk of anoxia appeared completely normal.

[Ca$^{2+}$] during normoxia, anoxia, and recovery. [Ca$^{2+}$] in cortical sheet neurons from room air-breathing turtles studied in oxygenated aCSF at 25°C was 135 ± 12 nM (n = 27). After 2 h of anoxia in vivo before brain dissection, [Ca$^{2+}$] increased to 164 ± 23 nM (n = 13, P < 0.05) and by 3 wk of anoxia had risen to 182 ± 12 nM (n = 5, P < 0.01 compared with control). However, at the end of 40 days of anoxia at 3°C, [Ca$^{2+}$] returned to approximately the same level as seen after 2 h of anoxia (Fig. 1). In turtles returned to room air after 4 wk of anoxia, [Ca$^{2+}$] returned to preanoxic levels within 24 h.

There was no difference in [Ca$^{2+}$] in cortical sheets taken from room air-breathing turtles kept at 25 or 3°C for 1–4 wk.

[Ca$^{2+}$], was also measured in cortical sheets bathed in aCSF containing the high Ca$^{2+}$ and high Mg$^{2+}$ levels and decreased pH present during prolonged dormancy (Fig. 1). In these slices, taken from turtles at ~1–wk intervals during 6 wk of anoxia, [Ca$^{2+}$] was higher than counterparts studied in anoxic standard Ca$^{2+}$ aCSF during weeks 1–4 (P < 0.05, unpaired t-tests), but were similar thereafter, despite a fivefold increase in extracellular Ca$^{2+}$ in the anoxic high-Ca$^{2+}$ aCSF.

NMDA receptor activity, NMDA receptor-mediated Ca$^{2+}$ changes (NMDA-ΔCa$^{2+}$) were measured as an index of receptor activity. In cortical sheets, NMDA-ΔCa$^{2+}$ decreased to 60 ± 14% of control after 2 h of anoxia (examples in Fig. 2). During 6 subsequent weeks of anoxia, NMDA-ΔCa$^{2+}$ was measured in slices bathed either in anoxic normal Ca$^{2+}$-aCSF (ionic composition and pH identical to normoxic turtles) or in anoxic high-Ca$^{2+}$ aCSF that was more acidic and contained greater amounts of Ca$^{2+}$ and magnesium (see Methods). In anoxic aCSF containing 1.3 mM Ca$^{2+}$, NMDA-ΔCa$^{2+}$ remained stably reduced at ~60% of preanoxic controls during 5 wk of anoxia (Fig. 3). In anoxic high-Ca$^{2+}$ aCSF, [Ca$^{2+}$] during normoxia, anoxia, and recovery. [Ca$^{2+}$] in cortical sheet neurons from room air-breathing turtles studied in oxygenated aCSF at 25°C was 135 ± 12 nM (n = 27). After 2 h of anoxia in vivo before brain dissection, [Ca$^{2+}$] increased to 164 ± 23 nM (n = 13, P < 0.05) and by 3 wk of anoxia had risen to 182 ± 12 nM (n = 5, P < 0.01 compared with control). However, at the end of 40 days of anoxia at 3°C, [Ca$^{2+}$] returned to approximately the same level as seen after 2 h of anoxia (Fig. 1). In turtles returned to room air after 4 wk of anoxia, [Ca$^{2+}$] returned to preanoxic levels within 24 h.

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aCSF, NMDA-ΔCa\textsuperscript{2+} responses were approximately twice controls during the first several weeks of anoxia (Figs. 2 and 3). However, after 3–6 wk, responses measured in this high-Ca\textsuperscript{2+}, high-Mg\textsuperscript{2+}, and low-pH medium were approximately equal to counterparts studied in standard aCSF. These changes represented a dramatic decrease in Ca\textsuperscript{2+} influx via the NMDA receptor in the presence of high extracellular Ca\textsuperscript{2+}. This is illustrated by a decrease in the ratio of NMDA receptor activity in high and low Ca\textsuperscript{2+} (Fig. 4) during the 6 wk of anoxia studied.

NMDA-ΔCa\textsuperscript{2+} returned to preanoxic control in cortical sheets from turtles after 24-h recovery in room air (at room temperature, ~25°C) after 4 wk of anoxia.

NMDA-ΔCa\textsuperscript{2+} in cortical sheets from normoxic turtles kept at 25°C was not different from that in sheets taken from normoxic turtles kept at 3°C for 4 wk.

**DISCUSSION**

I found that NMDA receptor activity in turtle cerebrocortex decreases during 6 wk of anoxia and that functional changes result in depressed Ca\textsuperscript{2+} permeability of the NMDA receptor ion channel despite a greater than four- to fivefold increase in CSF [Ca\textsuperscript{2+}] that probably occurs over this time. These changes may play important roles in decreasing neuronal excitability and neuronal energy expenditure and preventing excessive Ca\textsuperscript{2+} influx during anoxic dormancy. As far as I am aware, this is the first demonstration of such a long-term adaptive change in an ion channel in a dormant animal.

Reduction of NMDA receptor activity during anoxia occurred during a 2-h period of anoxia, and when measured under pH and ionic conditions like that of CSF in air-breathing turtles, this same degree of inactivation was present for 6 wk. NMDA receptor inactivation during the first several hours of anoxia is mediated by both adenosine receptors (4, 5) and phosphorylation of the NMDA receptor complex by neuronal phosphatases 1a or 2b (2). Therefore, other mechanisms (e.g., receptor internalization or removal from the plasma membrane) are not necessarily needed to explain the long-term receptor suppression during 5 additional weeks of anoxia. However, the results do not rule out long- or short-term changes in receptor number, nor do they imply that phosphorylation or actions of adenosine are necessarily the only mechanism by which receptor inactivation occurs.
Changes in NMDA receptor activity in the presence of the low-pH, high-Mg\textsuperscript{2+}, and high-Ca\textsuperscript{2+} conditions found in CSF during prolonged anoxic dormancy suggest a more complicated picture than simple single-step inactivation processes occurring at the beginning or end of anoxia. Apparently, the Ca\textsuperscript{2+} sensitivity of the NMDA receptor undergoes a continuing process of alteration during long-term dormancy, as suggested by the progressive decrease in the ratio of NMDA receptor activity in high and low Ca\textsuperscript{2+} during the 6-wk period of study (Fig. 4). Mammalian NMDA receptors are inhibited by low pH (22) and elevated Mg\textsuperscript{2+} (1, 22), whereas increased extracellular Ca\textsuperscript{2+} will increase Ca\textsuperscript{2+} influx via the receptor ion channel. I hypothesized that the low pH and high Mg\textsuperscript{2+} present in CSF during long-term anoxia would decrease the activity of the NMDA receptor to a significant degree compared with responses in 1.8 mM Ca\textsuperscript{2+}. Clearly, this hypothesis is incorrect, since NMDA-ΔCa\textsuperscript{2+} in this medium is about twice as great as in standard aCSF, even though [Ca\textsuperscript{2+}] is five times as great. NMDA-ΔCa\textsuperscript{2+} in high-Ca\textsuperscript{2+} aCSF decreased progressively during anoxia, suggesting that alterations in the Ca\textsuperscript{2+} permeability of the receptor occur during prolonged dormancy. The following processes could be involved in producing such long-term alterations in receptor function: 1) phosphorylation/dephosphorylation of regulatory sites on the cytoplasmic domains of receptor subunits; 2) allosteric modulation by regulatory factors including glycine, polyamines, protons, magnesium, and arachidonic acid; 3) changes in the number of NMDA receptors present in the cell membrane; and 4) expression of other NMDA receptor subtypes composed of different subunits. Hume et al. (12) found that single amino acid substitutions in glutamate receptor subunits markedly influence Ca\textsuperscript{2+} permeability. Further study will reveal which of these occur. It should be noted that numbers of NMDA receptors do not change in turtle forebrain during 2–6 h of anoxia (21).

[Ca\textsuperscript{2+}] increased ~22% during 2 h of anoxia, and continued to increase for 3–4 wk, before returning to control levels. [Ca\textsuperscript{2+}] could thus be an important messenger that initiates and sustains key regulatory processes that regulate ion channels such as the NMDA receptor. This hypothesis is supported both by the temporal relationship between the increase in [Ca\textsuperscript{2+}] and NMDA receptor activity and data showing that elevated [Ca\textsuperscript{2+}] inactivates the NMDA receptor. A Ca\textsuperscript{2+}-dependent inactivation of NMDA receptors has been documented to occur by 1) Ca\textsuperscript{2+}-dependent actin-NMDA receptor depolymerization (20) and 2) binding of Ca\textsuperscript{2+}-calmodulin to the cytoplasmic domains of NMDA receptor NR\textsubscript{1} subunits (24). It is also possible that elevated Ca\textsuperscript{2+} activates protein kinases or phosphatases via Ca\textsuperscript{2+}-calmodulin or other effector molecules. The actual role of Ca\textsuperscript{2+} in activating these processes in turtle neurons during anoxia will be defined in future studies.

Several methodological issues deserve comment. Estimates of [Ca\textsuperscript{2+}] with fura 2 are subject to a number of possible problems, as reviewed by Moore et al. (15). It is possible that chemical or physical changes in the turtle cortical neurons during anoxia alter the properties of fura 2, such as dye binding affinity. However, such changes in fura 2 Ca\textsuperscript{2+} binding properties are unlikely to significantly alter our conclusions, since very large changes in viscosity or ionic strength are required to alter fura 2 Ca\textsuperscript{2+} binding by >10% (15).

Another potential problem is the way in which we have estimated NMDA receptor activity by assuming that activity is estimated by Ca\textsuperscript{2+} changes (Ca\textsuperscript{2+} influx) stimulated by NMDA. Even though we have shown that activity measured this way correlates well with receptor activity changes measured with cell-attached...
patch-clamp measurements of receptor open probability (5), conditions occurring during prolonged anoxia could rupture this correlation. Future studies with direct measurements of ion channel activity (channel open probability measurements with patch clamping) will be needed to settle this issue.

Perspectives

Significance of NMDA receptor inactivation during prolonged hypoxia and dormancy. The significance of the downregulation of excitatory receptors during anoxia relates to three key events that contribute to the long-term survival of the turtle brain during anoxia: 1) reduction in the consumption of ATP required for maintenance of electrical excitability and neurotransmission; 2) a reversible reduction in neuron excitability, which is consistent both with behavioral inactivity during dormancy and with the capability of increases in neuronal activity at the end of a dive or at the termination of dormancy; and 3) decreased possibility of lethal \([Ca^{2+}]_i\) accumulation.

Suppression of NMDA receptor activity is probably a key event in the downregulation of neural activity that occurs in the turtle brain during anoxia. The NMDA receptor is an important ionotropic ion channel, being a member of the glutamate receptor family, which is the dominant excitatory neurotransmitter system in the vertebrate brain and spinal cord. It is reasonable to hypothesize that \(Ca^{2+}\) influx through this receptor channel would be subject to a variety of controls. It furthermore could be hypothesized that limiting \(Ca^{2+}\) influx through the NMDA receptor is critical for cell survival, since \(Ca^{2+}\) elevation is a primary cause of cell death from hypoxia and ischemia in mammalian neurons. Indeed, it is believed that \(Ca^{2+}\) plays a central role in necrotic and apoptotic cell death in a variety of mammalian and nonmammalian cells (7). A key factor in the adaptation of the brain of Chrysemys to anoxia is a profound reduction in metabolic rate, which must involve decreased pumping and leak rates of cations such as Na\(^+\) and \(Ca^{2+}\), since cation pumps account for most of the ATP consumed in nerve cells. Evidence shows that depression of ion channel activity does occur in turtles during anoxia, in both liver and brain. Buck and Hochachka (6) showed that ion pumping decreased markedly in turtle hepatocytes during anoxia, and Buckler and co-workers (3–5) demonstrated that glutamate receptors were inactivated in brain. Evidence for anoxia-induced inactivation of Na\(^+\) channels and K\(^+\) channels has been put forth by Lutz and co-workers (13, 17–19), changes which are apparently under the control of adenosine receptors.

A possible sequence of events that reversibly inactivates turtle NMDA receptors during anoxia is as follows. A change in metabolism from oxidative to anaerobic is accompanied by a drop in brain ATP of \(-20%\) and a decrease in brain intracellular pH of \(-0.6\) units (L. T. Buck and P. E. Bickler, unpublished data). The decrease in intracellular pH increases \([Ca^{2+}]\), by releasing \(Ca^{2+}\) from \(Ca^{2+}\)-binding proteins. A sequence of \(Ca^{2+}\)-dependent events then ensues. \(Ca^{2+}\) binds to actin, resulting in actin-NMDA depolymerization and decreased NMDA receptor open probability (20). \(Ca^{2+}\) also binds to \(Ca^{2+}\)-binding proteins such as calmodulin, which triggers activation of protein kinases and phosphatases that lead to phosphorylation/dephosphorylation events at the intracellular loops of the NMDA receptor. These changes decrease open channel probability. Another factor decreasing NMDA receptor activity is adenosine. Adenosine, an ATP metabolite accumulating transiently in the brain during anoxia (16), decreases NMDA-\(\Delta Ca^{2+}\) and single-channel NMDA receptor open probability in turtle cerebrotectical neurons by acting via adenosine receptors (4, 5, 20). Prolonged anoxia (weeks to months) brings other changes: further allosteric modulation of the NMDA receptor or assembly of NMDA receptors with different subunits. Although protein synthesis is reduced substantially during anoxia (11), alterations in receptor composition may be the result of changes in the membrane half-life of one or another subunit/receptor subtype.

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REFERENCES


