Reduction of NMDA receptor activity in cerebrocortex of turtles (Chrysemys picta) during 6 wk of anoxia

Bickler, P. E.  Reduction of NMDA receptor activity in cerebrocortex of turtles (Chrysemys picta) during 6 wk of anoxia. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R86–R91, 1998.—Survival of brain anoxia during months of winter dormancy by the Western painted turtle, Chrysemys picta, may rely on inactivation of neuronal ion channels. During 2 h of anoxia, Ca2+ influx via the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor decreases 30–40%, but it is not known if prolonged anoxic dormancy is associated with even more profound downregulation of this important channel. Because ionized Ca2+ in cerebrospinal fluid (CSF) increases five- to sixfold during prolonged anoxia, the potential for uncontrolled Ca2+ influx and neurotoxicity is increased. To study the regulation of NMDA receptor activity, we measured NMDA-mediated changes in intracellular Ca2+ (NMDA-ΔCa2+) in turtle cerebrocortical sheets with fura 2. Turtles were kept in N2-bubbled aquariums for 2 h to 6 wk at 2–3°C. NMDA-ΔCa2+ decreased 60 ± 14% (P < 0.05) after 2 h of anoxia and did not decrease further for 6 wk. Intracellular Ca2+ increased from 135 to 183 nM (P < 0.05) after 3 wk of anoxia and thereafter returned toward preanoxic levels. When NMDA receptor activity was assessed in artificial CSF containing the ions found in anoxic brain CSF (pH 7.25, 69 mM lactate, 8.4 mM Ca2+, and 5.1 mM Mg2+), NMDA-ΔCa2+ was twice control initially but was 21% less than in normoxic artificial CSF after the end of 6 wk, suggesting altered sensitivity of the NMDA receptor to ionized Ca2+ during prolonged anoxia. Regulation of NMDA receptor activity in turtle cerebrocortex during 6 wk of anoxia thus results in depression of NMDA receptor Ca2+ flux, despite a sixfold increase in ionized extracellular Ca2+.

turtle; N-methyl-D-aspartate receptor; fura 2; receptor downregulation; anoxia-tolerant neurons; dormancy; calcium; magnesium

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). 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 submersion 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 belli) 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 fura 2. The methods for dissecting, loading slices with fura 2, and measuring intracellular Ca$^{2+}$ changes are exactly as described in Buck and Bickler (4). Cortical sheets from anoxic turtles were dissected and fura 2-loaded in a nitrogen atmosphere (Atmos bag, Fisher Scientific). Four to six sheets were obtained from each animal. During fura loading, slices were continuously bubbled with 95% N2 and 3% CO$_2$. 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, slices 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$^{2+}$]i) 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$_3^-$, bubbled with either 95% O$_2$ and 5% CO$_2$ or 95% N2 and 5% CO$_2$. There was no difference in [Ca$^{2+}$]i during normoxia, anoxia, and recovery. [Ca$^{2+}$]i during normoxia, anoxia, and recovery. [Ca$^{2+}$]i 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$^{2+}$]i 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-$\Delta$Ca$^{2+}$) were measured as an index of receptor activity. In cortical slices, NMDA-$\Delta$Ca$^{2+}$ decreased to 60 ± 14% of control after 2 h of anoxia (examples in Fig. 2). During 6 subsequent weeks of anoxia, NMDA-$\Delta$Ca$^{2+}$ 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-$\Delta$Ca$^{2+}$ remained stably reduced at ~60% of preanoxic controls during 5 wk of anoxia.

**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.

Fig. 1. Intracellular Ca$^{2+}$ levels in turtle cerebrocortex. Cortical tissue was obtained from turtles during 2 h to 6 wk of anoxia in vivo. Intracellular Ca$^{2+}$ levels ([Ca$^{2+}$]i) were measured in tissue bathed in either artificial cerebrospinal fluid (aCSF) containing ions present in normoxic turtles (1.3 mM Ca$^{2+}$) or in turtle aCSF containing 8.4 mM Ca$^{2+}$ (found in CSF after 6–8 wk of dormancy; see text for other ions present). *Significant difference compared with 2-h anoxia group for 1.3 mM Ca$^{2+}$ conditions. †Significant difference for 8.4 mM Ca$^{2+}$ conditions.
aCSF, NMDA-\(\Delta Ca^{2+}\) 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\(^{2+}\), high-Mg\(^{2+}\), and low-pH medium were approximately equal to counterparts studied in standard aCSF. These changes represented a dramatic decrease in Ca\(^{2+}\) influx via the NMDA receptor in the presence of high extracellular Ca\(^{2+}\). This is illustrated by a decrease in the ratio of NMDA receptor activity in high and low Ca\(^{2+}\) (Fig. 4) during the 6 wk of anoxia studied.

NMDA-\(\Delta Ca^{2+}\) 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-\(\Delta Ca^{2+}\) 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\(^{2+}\) permeability of the NMDA receptor ion channel despite a greater than four- to fivefold increase in CSF [Ca\(^{2+}\)] that probably occurs over this time. These changes may play important roles in decreasing neuronal excitability and neuronal energy expenditure and preventing excessive Ca\(^{2+}\) 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$^{2+}$, and high-Ca$^{2+}$ 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$^{2+}$ 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$^{2+}$ during the 6-wk period of study (Fig. 4). Mammalian NMDA receptors are inhibited by low pH (22) and elevated Mg$^{2+}$ (1, 22), whereas increased extracellular Ca$^{2+}$ will increase Ca$^{2+}$ influx via the receptor ion channel. I hypothesized that the low pH and high Mg$^{2+}$ 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$^{2+}$. Clearly, this hypothesis is incorrect, since NMDA-Na$^{+}$ in this medium is about twice as great as in standard aCSF, even though [Ca$^{2+}$] is five times as great. NMDA-Na$^{2+}$ in high-Ca$^{2+}$ aCSF decreased progressively during anoxia, suggesting that alterations in the Ca$^{2+}$ 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$^{2+}$ 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$^{2+}$], increased ~22% during 2 h of anoxia, and continued to increase for 3–4 wk, before returning to control levels. [Ca$^{2+}$] 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$^{2+}$] and NMDA receptor activity and data showing that elevated [Ca$^{2+}$], inactivates the NMDA receptor. A Ca$^{2+}$-dependent inactivation of NMDA receptors has been documented to occur by 1) Ca$^{2+}$-dependent actin-NMDA receptor polymerization (20) and 2) binding of Ca$^{2+}$-calmodulin to the cytoplasmic domains of NMDA receptor NR$_2$ subunits (24). It is also possible that elevated Ca$^{2+}$ activates protein kinases or phosphatases via Ca$^{2+}$-calmodulin or other effector molecules. The actual role of Ca$^{2+}$ in activating these processes in turtle neurons during anoxia will be defined in future studies.

Several methodological issues deserve comment. Estimates of [Ca$^{2+}$] 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$^{2+}$ 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$^{2+}$ 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$^{2+}$ changes (Ca$^{2+}$ 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 [Ca2+1 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 Ca2+ influx through this receptor channel would be subject to a variety of controls. It furthermore could be hypothesized that limiting Ca2+ influx through the NMDA receptor is critical for cell survival, since Ca2+ elevation is a primary cause of cell death from hypoxia and ischemia in mammalian neurons. Indeed, it is believed that Ca2+ 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 Ca2+, 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 Bickler 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 [Ca2+1 by releasing Ca2+ from Ca2+ binding proteins. A sequence of Ca2+-dependent events then ensues. Ca2+ binds to actin, resulting in actin-NMDA depolymerization and decreased NMDA receptor open probability (20). Ca2+ also binds to Ca2+ 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-ΔCa2+1 and single-channel NMDA receptor open probability in turtle cerebrocortical 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-induced inactivation of Na+ channels and K+ channels (13, 17–19), depression of ion channel activity does occur in turtle hepatocytes during anoxia. Further studies with open probability measurements with patch clamping) will be needed to settle this issue.

**REFERENCES**