Brain Research, 625 (1993) 181–185

BRES 19281

Research Reports

Unchanged [^{3}H]MK-801 binding and increased [^{3}H]flunitrazepam binding in turtle forebrain during anoxia

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(Accepted 11 May 1993)

Key words: Glutamate; γ-Aminobutyric acid; *N*-Methyl-D-aspartate; Dizocilpine; Excitotoxicity; Excitatory amino acid; Ischemia

In order to determine if functional changes in *N*-methyl-D-aspartate receptors and $GABA_A$ receptors play a role in the remarkable anoxia tolerance of freshwater turtle brain, we used autoradiographic techniques to assay [^{3}H]MK-801 and [^{3}H]flunitrazepam binding in turtle forebrain after turtles had been subjected to anoxia for 2 or 6 h. The effects of glutamate, glycine, competitive *N*-methyl-D-aspartate antagonists, glycine antagonists, polyamines, magnesium, and zinc on [^{3}H]MK-801 binding were the same in anoxic and control turtle forebrains. These results indicate that NMDA receptor regulation plays no role in the adaptive responses to anoxia in turtle brain. In contrast, [^{3}H]flunitrazepam binding was significantly increased in the anoxic dorsal cortex and striatum. The most parsimonious explanation for elevated benzodiazepine receptor binding is that the rise in extracellular GABA levels known to accompany anoxia enhances benzodiazepine receptor affinity. It is possible, however, that $GABA_A$ receptor upregulation during anoxia increases the effectiveness of the inhibitory action of released GABA and contributes to the anoxia tolerance of turtles.

INTRODUCTION

The mammalian central nervous system is intolerant of anoxia. Even minutes of exposure to anoxia can be sufficient to cause energy failure, loss of ionic gradients, and depolarization. A series of cell damaging events ensues, including massive release of the excitatory amino acids (EAAs) glutamate and aspartate from presynaptic terminals[3,8,15,19]. The overstimulation of one subtype of EAA receptor, the *N*-methyl-D-aspartate (NMDA) receptor, has been particularly implicated in anoxic brain injury. Depolarization allows glutamate to activate the NMDA receptor by removing the voltage dependent Mg^{2+} block, thereby opening Ca^{2+} permeable channels and producing a flood of Ca^{2+} ions into the cell from the extracellular fluid[3,15,19]. The uncontrolled rise in intracellular calcium is felt to be an important factor in neuronal injury during anoxia.

Although this vulnerability of the brain to anoxia is a common feature of vertebrate brain, there are exceptions. Some species of freshwater turtles, for example, have brains that can withstand complete anoxia for days without any adverse sequalae[6]. The mechanisms underlying the turtle brain avoidance of anoxia related catastrophies are of interest to those concerned with biological strategies for brain survival when oxygen supply is limited. A basic premise is that the anoxic turtle brain is able to suppress its metabolic demand to a level that can be satisfied by a reduced anaerobic glycolysis. A variety of factors appear to be involved in achieving this hypometabolic state and neurotransmitters are likely to play a key role in these processes. In particular, the action of inhibitory and excitatory amino acid neurotransmitters may play a crucial role in the adaptation of turtle brain to anoxia[6,7,9,10].

The turtle brain displays dramatic physiological compensations during anoxia; supression of synaptic activity[6], down-regulation of sodium channels[13], sustained release of inhibitory neurotransmitters and (in sharp contrast to the mammalian brain) extracellular

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EAA concentrations are not increased during many hours of anoxia[6,7,9,10]. Turtle neurons may also have a decreased sensitivity to excitotoxic injury[20,21]. This could be due to intrinsic differences in the function of EAA receptors, particularly NMDA receptors. Recent studies, however, show that in the normoxic turtle forebrain, NMDA receptors have the same pharmacological characteristics as NMDA receptors in mammalian brain[16]. In view of the substantial protective changes that occur in turtle brain during anoxia it was thought of interest to determine if anoxia also induced functional changes in inhibitory and excitatory receptors in the turtle brain. NMDA receptors were assessed with [^{3}H]MK-801 (dizocilpine) binding, a phencyclidine (PCP) ligand that binds within the NMDA receptor ionophore. The amount of [^{3}H]MK-801 binding is regulated by the degree of activation of other sites on the NMDA receptor complex, making [^{3}H]MK-801 binding a probe of NMDA receptor function[2,17,22]. The status of $GABA_A$ receptors during anoxic conditions was probed with the benzodiazepine (BDZ) ligand [^{3}H]flunitrazepam.

MATERIALS AND METHODS

Materials

Experiments were performed on freshwater turtles *Trachemys* (formerly *Pseudemys*) *scripta* obtained from a local supplier. [^{3}H]MK-801 and [^{3}H]flunitrazepam were obtained from DuPont-NEN (Boston, MA). Non-radioactive MK-801 was a gift of Dr. L.L. Iversen (Merck, Sharpe & Dohme; Essex, UK). Clonazepam was a gift of Dr. Peter Sorter (Hoffman-LaRoche; Nutley, NJ). 3-(2-Carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) and 7-chlorokynurenic acid (7-ClK) were purchased from Cambridge Research Biochemicals (Wilmington, DE). All other reagents were purchsed from Sigma (St. Louis, MO).

Brain preparation

Anoxia was induced as described previously[9]. Six animals were subjected to 6 h of anoxia, 6 animals were subjected to 2 h of anoxia and 6 animals were treated as controls. At the end of the anoxic period, animals were decapitated, the brains extracted from the cranial vault, coated with Lipshaw embedding matrix and frozen in crushed dry ice. Brains were sent via overnight mail from Miami to Ann Arbor surrounded by crushed dry ice in an insulated package. Upon arrival, the brains were stored at −70°C until time of assay.

[^{3}H]MK-801 binding

[^{3}H]MK-801 binding was assessed with the technique of Sakurai et al.[17]. Briefly, brains were warmed to −20°C and 20 μm sections were cut on a Lipshaw cryostat, thaw-mounted onto gelatin-coated slides, and stored at −20°C until time of assay (24–48 h). Duplicate sections were prewashed in 50 mM Tris-Acetate buffer (pH 7.4, 4°C) for 30 min, dried under a stream of cool air, and immersed in buffer containing 5 nM [^{3}H]MK-801 and other compounds for 120 min at room temperature. Following incubation, sections were rinsed in buffer (4°C) for 80 min and dried under a stream of hot air. Non-specific binding was assessed with the addition of 5 μM MK-801.

Regulation of [^{3}H]MK-801 binding by other sites on the NMDA receptor/ionophore complex was studied in two complementary ways. To analyze the effects of activating the EAA, strychine-insensitive glycine, and polyamine sites, [^{3}H]MK-801 binding was studied in the absence and presence of 30 μM glutamate, 10 μM glycine, 30 μM glutamate/10 μM glycine, 100 μM spermine, 100 μM spermidine, 100 μM arcaine, and 100 μM spermine/100 μM arcaine. As an additional means of studying the modulation of [^{3}H]MK-801 binding by the EAA and strychnine-insensitive glycine sites, [^{3}H]MK-801 binding was assessed in the presence of varying concentrations of the NMDA competitive antagonist CPP, and the strychnine-insensitive glycine antagonist 7ClK. In these experiments, 10 μM glycine and 30 μM glutamate were added to the incubation solution to compensate for the fact that competitive NMDA antagonists and glycine antagonists slow the approach to equilibrium of [^{3}H]MK-801 binding. The effects of polyamine agonists, the polyamine antagonist arcaine, and the effects of zinc and magnesium were also assessed in the presence of 10 μM glycine/30 μM glutamate.

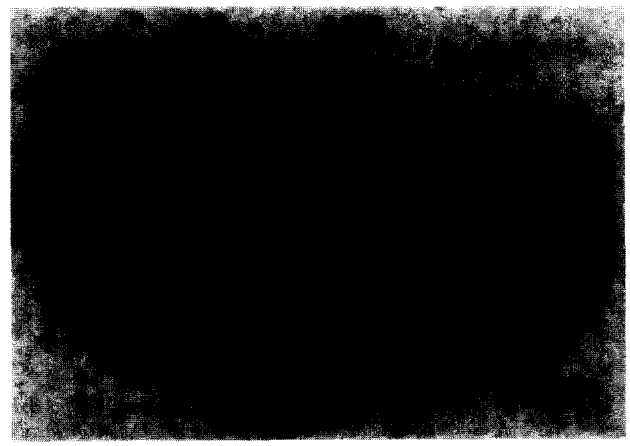

Fig. 1. Autoradiograph of [^{3}H]MK801 binding in turtle forebrain. Abundant binding is present in the dorsal cortex (CTX), hippocampus (HIP), and dorsal ventricular ridge (DVR). Lower levels of binding are found in the striatum (STR).

[^{3}H]Flunitrazepam binding

[^{3}H]Flunitrazepam binding was assessed as described previously[1]. Sections were prewashed in 50 mM Tris-acetate buffer (pH 7.2, 4°C) for 30 min, dried under a stream of cool air, and immersed in ligand solution containing 5 nM [^{3}H]flunitrazepam. Incubation was terminated with one quick dip in buffer followed by 2×5 minute washes in buffer (4°C), and sections were dried under a stream of hot air. Non-specific binding was assessed with the addition of 2 μM clonazepam.

Data analysis

Slides were apposed to tritium sensitive film (Hyperfilm, Amersham) and exposed along with standards containing known amounts of radioactivity (ARC, St. Louis, Mo) for 1–3 weeks. Films were developed in D-19 (Kodak) and binding levels quantitated with computer assisted densitometry using the MCID system (Imaging Research, St. Catherines, ONT). Regions analyzed included the dorsal ventricular ridge (DVR, Fig. 1), the striatum (STR, Fig. 1), the dorsal cortex (CTX, Fig. 1), and the hippocampus (HIP, Fig. 1). Regions were ascertained by referring to the atlas of Powers and Reiner[15]. Values from duplicate sections were averaged to yield a single value for each animal under each condition.

Statistical comparisons were made between the control, 2 h of anoxia, and 6 h of anoxia groups with a one-way ANOVA followed by Fisher's PLSD test for post-hoc comparisons using the Statview II program (Abacus Concepts, Berkeley, CA).

RESULTS

[^{3}H]MK-801 binding

Results were identical in all regions. To avoid redundancy, most data presented is derived from analysis of the dorsal cortex.

TABLE I

Regional distribution of [^{3}H]MK-801 binding sites (pmol / mg protein) in control and anoxic turtle brains

Values represent mean ± S.E.M. of 3 turtles in each condition as described in text. [^{3}H]MK-801 binding was performed under basal binding conditions (no exogenous glutamate or glycine).

Region	*Control*	*2 Hour*	*6 Hour*
STR	0.13 ± 0.02	0.18 ± 0.02	0.13 ± 0.02
DVR	0.23 ± 0.02	0.26 ± 0.03	0.23 ± 0.07
DC	0.21 ± 0.02	0.19 ± 0.03	0.17 ± 0.02
HIP	0.24 ± 0.02	0.23 ± 0.02	0.22 ± 0.02

Under basal binding conditions (no additional glutamate or glycine) there was no significant difference between anoxic and control animals in [^{3}H]MK-801 binding (Table I, Fig. 2). Addition of 30 μM glutamate or 10 μM glycine or 30 μM glutamate/10 μM glycine did not increase [^{3}H]MK-801 binding. Following the addition of 30 μM glutamate or 10 μM glycine (Fig. 2), there was also no difference between [^{3}H]MK-801 binding in anoxic and normoxic turtle forebrain. Similarly, while addition of the polyamine agonists spermine and spermidine increased [^{3}H]MK-801 binding, there was no difference between anoxic and control animals (Fig. 2). [^{3}H]MK-801 binding was decreased by the polyamine antagonist arcaine to an equal extent in anoxic and control animals and arcaine antagonized the stimulatory effects of spermine (Fig. 2).

In the presence of 30 μM glutamate and 10 μM glycine, CPP and 7ClK inhibited [^{3}H]MK-801 binding. There was no difference in the degree of inhibition between control or anoxic groups of animals (Fig. 3).

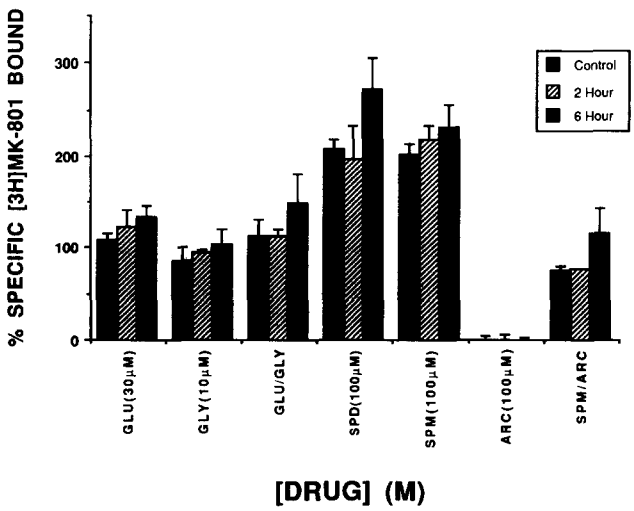

Fig. 2. The effects of added glutamate (GLU), glycine (GLY), glutamate and glycine (GLU/GLY), spermidine (SPD), spermine (SPM), arcine (ARC), and spermine plus cortex binding and normalized to basal (no glutamate nor glutamate and glycine. There is no effect of anoxia under any condition. Bars are mean ± S.E.M., $n = 3$ animals for each condition.

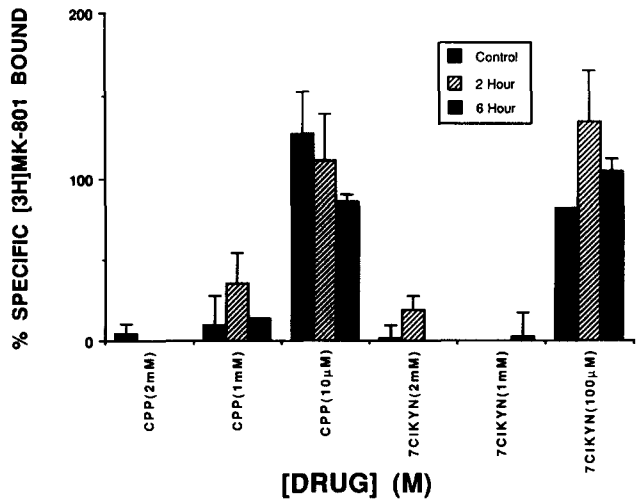

Fig. 3. The effects of CPP and 7ClK on [^{3}H]MK-801 binding in dorsal cortex. Both compounds inhibit binding and there is no effect of anoxia. Experiments performed in the presence of 30 μM glutamate/10 μM glycine (see text for details). Bars are mean ± S.E.M., $n = 3$ animals for each condition.

Zinc and magnesium, in the presence of 30 μM glutamate/10 μM glycine, also inhibited [^{3}H]MK-801 binding without any significant differences between control and anoxic groups (Fig. 4). In the presence of 30 μM glutamate/10 μM glycine, spermine and spermidine continued to stimulate [^{3}H]MK-801 binding and these effects, as well as unstimulated binding, were inhibited by the polyamine antagonist arcaine (Fig. 5).

[^{3}H]Flunitrazepam

In contrast to the lack of change in [^{3}H]MK-801 binding, [^{3}H]flunitrazepam binding was enhanced in some brain regions during anoxia. After both 2 and 6 h

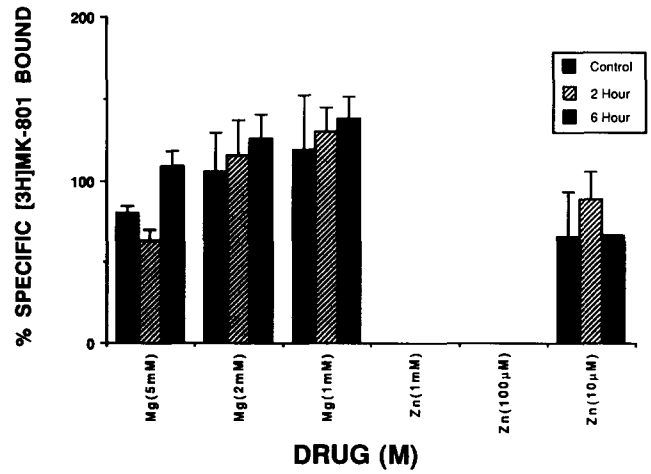

Fig. 4. The effects of zinc and magnesium on [^{3}H]MK-801 binding in dorsal cortex. Experiments performed in the presence of 30 μM glutamate/10 μM glycine. Both ions produce inhibition of binding and there is no effect of anoxia. Bars are mean ± S.E.M., $n = 3$ animals for each condition.

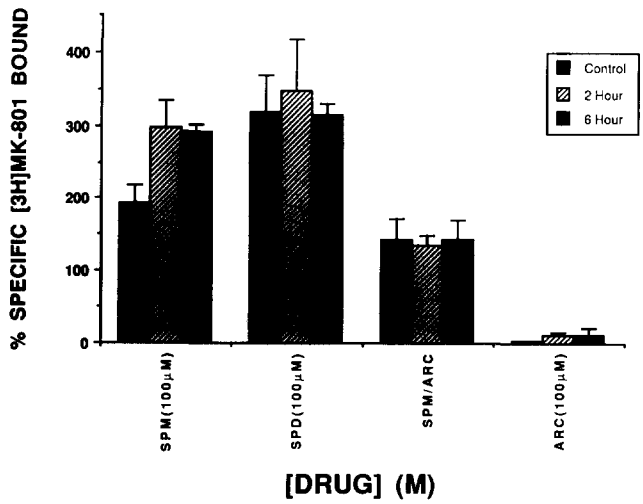

Fig. 5. The effects of spermine (SPM), spermidine (SPD), and arcaine (ARC) on [^{3}H]MK-801 binding in dorsal cortex in the presence of 30 μM glutamate/10 μM glycine (see text for details). There is no effect of anoxia. Bars are mean $\pm$ S.E.M., $n = 3$ animals for each condition.

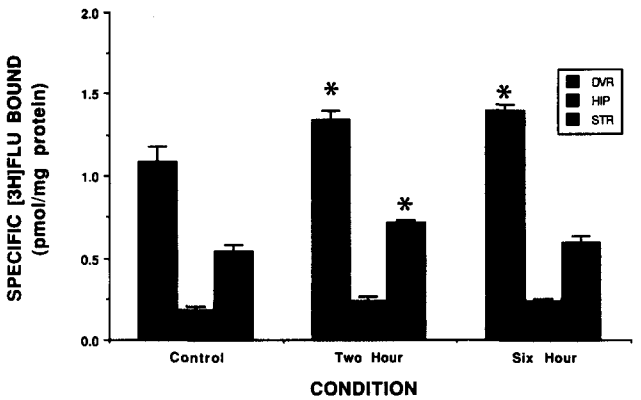

Fig. 6. The effect of anoxia on benzodiazepine binding sites in the turtle forebrain. Binding is elevated in the dorsal ventricular ridge after 2 and 6 h of anoxia and in the striatum after 2 h of anoxia. * = $P < 0.03$ by ANOVA followed by post-hoc Scheffe's test. Bars are mean $\pm$ S.E.M., $n = 3$ animals for each condition.

of anoxia, [^{3}H]flunitrazepam binding was enhanced in the DVR (Fig. 6). [^{3}H]Flunitrazepam binding was also increased in the STR after 2 h of anoxia (Fig. 6).

DISCUSSION

As indicated by the lack of change in PCP binding site pharmacology over 6 h of anoxia, the NMDA receptor appears to function normally during anoxia in turtles.

The binding of PCP ligands such as [^{3}H]MK-801 to the NMDA receptor is regulated by the activation of other sites on the NMDA receptor and this property makes [^{3}H]MK-801 binding a good probe of NMDA receptor function[2,17,22]. In well washed homogenate preparations, [^{3}H]MK-801 binding is enhanced by addition of glutamate and glycine agonists[2,22]. This effect is not observed in autoradiographic preparations, possibly due to the presence in tissue sections of sufficient endogenous glutamate and glycine to stimulate [^{3}H]MK-801 binding[17]. Polyamine agonists enhance and polyamine antagonists inhibit [^{3}H]MK-801 binding in both homogenate and autoradiographic preparations[2]. In the present experiments and prior studies, we found that addition of glutamate and glycine to turtle tissue sections had no effect on [^{3}H]MK-801 binding under normoxic conditions. Polyamine agonists enhanced [^{3}H]MK-801 binding and this enhancement, as well as basal [^{3}H]MK-801 binding, was inhibited by the polyamine antagonist arcaine. There was no difference in the effects of glutamate, glycine, or polyamine compounds between normoxic and anoxic animals.

As an additional means of evaluating the linkage between the EAA and strychnine-insensitive glycine sites and the [^{3}H]MK-801 binding site, the effects of CPP and 7ClK on [^{3}H]MK-801 binding were evaluated in the presence of 30 μM glutamate and 10 μM glycine. Competitive NMDA and glycine antagonists diminish [^{3}H]MK-801 binding. The effects of these antagonists were evaluated in the presence of additional glutamate and glycine because NMDA and glycine antagonists slow the attainment of equilibrium binding by PCP ligands, necessitating the addition of exogenous glutamate and glycine to ensure the presence of equilibrium binding conditions. In the present experiments, CPP and 7ClK inhibited [^{3}H]MK-801 binding equally under normoxic and anoxic conditions.

The effects of polyamine compounds were also studied in the presence of 30 μM glutamate/10 μM glycine. Polyamine agonists stimulated [^{3}H]MK-801 binding while the polyamine antagonist arcaine inhibited both basal and polyamine agonist stimulated [^{3}H]MK-801 binding. These results were found in both normoxic and anoxic states. The effects of the divalent cations magnesium and zinc, which possess separate inhibitory sites on the NMDA receptor complex and inhibit [^{3}H]MK-801 binding, were also assayed in the presence of 30 μM glutamate/10 μM glycine. Both these divalent cations inhibited [^{3}H]MK-801 binding equally in normoxic and anoxic brains.

Our results indicate that there are no qualitative differences in the function of NMDA receptors during anoxia in turtle forebrain. The coupling of EAA agonist, strychnine-insensitive glycine, polyamine, magnesium, and zinc sites to the PCP binding site is preserved during anoxia in turtle brain. However, because we assayed the effect of a number of compounds on [^{3}H]MK-801 binding with a limited number of concen-

trations of each substance in a small number of brains, we cannot exclude the possibility that there is a quantitative difference in the coupling of the EAA agonist, strychnine-insensitive glycine, polyamine, magnesium, or zinc sites to the PCP binding site.

Our results do not exclude a role for interspecies differences in the function of EAA receptors during anoxia as an adaptive mechanism in turtle brain. While much of the literature on the role of EAAs in anoxia has concentrated on NMDA receptors, recent data indicates that activation of the α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid/kainate (AMPA/KA) subtype of EAA receptors may play a role in neuronal damage during hypoxia/ischemia. Selective AMPA antagonists are neuroprotective in some models of hypoxia/ischemia and several studies have shown that some AMPA/KA receptors are capable of fluxing Ca^{2+} (refs. 5, 12, 18). Studies of turtle brain AMPA/KA receptors may be rewarding.

In contrast to the lack of change in [^{3}H]MK-801 binding, [^{3}H]flunitrazepam binding to the $GABA_A$ receptor was increased in the DVR and STR of the turtle brain within 2 hr of anoxia. Elevated BDZ receptor binding occurs also in mammalian brain during hypoxia, and increased extracellular GABA levels are known to accompany hypoxia/ischemia in rat brain[4,11]. The most parsimonious explanation for elevated BDZ binding during anoxia is that the rise in extracellular GABA which occurs during anoxia enhances BDZ receptor affinity. It is possible, however, that the $GABA_A$ receptor is upregulated during anoxia, thereby increasing the effectiveness of the inhibitory action of released GABA.

Further study of neurotransmitter receptor function in anoxic turtle brain may lead to new insights into the pathophysiology of anoxic brain injury.

Acknowledgements. Supported by Grants NS19613 and NS01300 to R.L.A. and IBN-91-21385 to P.L.L.

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