Functional NMDA Receptor Subtype 2B Is Expressed in Astrocytes after Ischemia *In Vivo* and Anoxia *In Vitro*

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NMDA-type glutamate receptors play a critical role in neuronal synaptogenesis, plasticity, and excitotoxic death. Recent studies indicate that functional NMDA receptors are also expressed in certain glial populations in the normal brain. Using immunohistochemical methods, we detected the presence of the NMDA receptor 2B (NR2B) subunit of the NMDA receptor in neurons but not astrocytes in the CA1 and subicular regions of the rat hippocampus. However, after ischemia-induced neuronal death in these regions, double immunohistochemical labeling revealed that NR2B subunits colocalized with the astrocyte marker glial fibrillary acid protein and with NR1 subunits that are required for functional NMDA receptors. NR2B expression was first observed 3 d after ischemia and reached a peak at 28 d. At 56 d, only a few NR2B-expressing astrocytes were still present. *In vitro*, when postnatal hippocampal cultures were subjected to 5 min of anoxia, it resulted in NR2B expression on astrocytes in the glial feed layer. Imaging of intracellular calcium with postanoxic cultures and astrocytes isolated acutely from the ischemic hippocampus revealed a rise in intracellular $[Ca^{2+}]$ after stimulation with the specific agonist NMDA. The response could be blocked reversibly with the competitive antagonist 2-amino-5-phosphonovalerate and attenuated by the NR2B-selective antagonist ifenprodil. Control astrocytes were not responsive to NMDA but responded to glutamate. An understanding of the role of astrocytes that express functional NMDA receptors in response to ischemia may guide development of novel stroke therapies.

Key words: ischemia; anoxia; NMDA; astrocytes; NR2B; calcium

Introduction

Investigations of the mechanism underlying neuronal death after ischemia and stroke have revealed a cascade of responses that result in neuronal death (Lipton, 1999). In the case of transient (usually ~12 min) forebrain or global ischemia, it is clear that some brain structures are more susceptible to ischemic injury than others and that even within a common structure, there are different levels of susceptibility between different populations of neurons. In the hippocampal formation, for example, ischemia results in neuronal death that is limited primarily to the pyramidal cells of the dorsal CA1 and subicular regions, with CA3 pyramidal cells and dentate granule cells being relatively spared (Freund et al., 1990; Sugawara et al., 1999).

In the normal brain, glial cells perform many functions that contribute to the survival of neurons, including catabolism of oxygen free radicals, uptake of neurotransmitters, and production and release of growth factors (Amédée et al., 1997; Tsacopoulos et al., 1997; Vannucci et al., 1997; Wiesinger et al., 1997). After brain injury, glia undergo characteristic changes that are thought to compose the inflammatory response to injury, and the

pattern of the glial response is a consistent and stereotypical feature in almost all types of brain pathology (Raivich et al., 1999). Astrocytes are activated within 2–4 d by a process that may include an action of cytokines (Stoll et al., 1998). These reactive astrocytes increase their glycolytic capacity, upregulate immediate early genes (Marrif and Juurlink, 1999), and express cytoskeletal elements such as glial fibrillary acid protein (GFAP) (Brenner, 1994), vimentin (Petito et al., 1990), and nestin (Lin et al., 1995), the latter two being cytoskeletal elements that are normally found only in immature glial cells (Messam et al., 2000; Sultana et al., 2000). After transient global ischemia in rats, reactive astrocytes are limited primarily to the CA1 and subicular regions of the hippocampus, in which there is a marked loss of pyramidal neurons (Stoll et al., 1998).

Aside from their important role in removing glutamate from the extracellular medium, astrocytes have been shown to respond to glutamate via activation of specific receptors. Glutamate acting on non-NMDA receptors (NMDARs) generates an inward current and an increase in cytosolic calcium that can spread throughout the glial syncytium linked by gap junctions (Glaum et al., 1990; Parpura et al., 1994; Porter and McCarthy, 1995, 1996; Pasti et al., 1997). The role of NMDARs in glial glutamate-mediated signaling has also been the focus of recent research. Functional NMDARs require NMDA receptor 1 (NR1) subunits in combination with NR2A–D or NR3 (Collingridge and Watkins, 1994; Petralia et al., 1994; Brimecombe et al., 1997; Dingledine et al., 1999). The NR2 subunits show distinct spatiotemporal distributions and modulate channel function (Varney et al., 1996; Scherzer et al., 1998; Sprengel et al., 1998; Chen et al., 1999; Hoffman et al., 2000; Sun et al., 2000; Lynch and Guttman, 2002). The pres-

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ence of NR2B has been identified in Bergmann glia of the cerebellum by *in situ* hybridization techniques (Luque and Richards, 1995), but electrophysiological studies of NMDARs in Bergmann glia and Mueller cells of the retina suggest that they are substantially different from NMDARs characterized in neurons (Muller et al., 1993; Uchihori and Puro, 1993). A more recent study demonstrates clearly the presence of functional NMDARs in astrocytes of the mouse neocortex (Schipke et al., 2001). In addition, the expression of NMDARs in astrocytes after ischemia, with an antibody that did not distinguish between NMDAR subtypes, has been reported previously (Gottlieb and Matute, 1997). Here, we report that both transient global forebrain ischemia *in vivo* and anoxia *in vitro* result in the specific expression of NR2B subunits in astrocytes and that their colocalization with NR1 subunits allows for the expression of functional NMDARs.

Materials and Methods

All procedures were in accordance with the guidelines established by the Canadian Council on Animal Care, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Postnatal hippocampal neuronal cultures. Hippocampal neuronal and glial cultures were prepared from 2- to 4-d-old Wistar rats as described previously (Abdel-Hamid and Baimbridge, 1997). Briefly, hippocampi were dissected from the brains, and the cells were dissociated and plated at a density of 3×10^5 neurons/cm² on poly-D-lysine- and laminin-coated coverslips in DMEM and F-12 (Invitrogen, San Diego, CA) supplemented with 10% (v/v) FBS (Invitrogen). After the first 24 hr, the medium was replaced completely by neurobasal medium containing B27 supplement, penicillin, and streptomycin. Cytosine arabinoside (10 μ M) was added 48 hr after initial plating to limit glial cell proliferation, and nutrients were replenished by replacing half the medium with fresh neurobasal and B27 twice weekly. All experiments were performed on primary cultures after 8–10 d *in vitro*.

Postnatal hippocampal astrocyte cultures. Hippocampi were dissected from the brains of 2- to 4-d-old Wistar rats, and the cells were dissociated and plated at a density of 1.5×10^6 cells/ml in ventilated 75 cm² flasks in basal Eagle's medium containing 15% FBS, glutamine, glucose, penicillin, and streptomycin (c-BME). When the cells had reached confluence, the flasks were shaken vigorously by hand to remove all other types of glia (McCarthy and de Vellis, 1980). The cells were then split and replated in c-BME. These passaged astrocytes were used for experiments when they had reached confluence.

Anoxia: neuronal and glial cell cultures. We used an anoxia model adapted from that described previously (Diarra et al., 1999). Cultures were removed from their growth medium, rinsed gently by immersion in a bicarbonate-buffered balanced salt solution (BBSS) that contained (in mM): NaCl 127, KCl 3, NaHCO₃ 19.5, NaH₂PO₄ 1.5, MgSO₄ 1.5, D-glucose 17.5, and CaCl₂ 1, and placed for 5 min at 37°C in fresh BBSS under 5% CO₂ and 95% O₂ or in BBSS previously gassed for 5 min with 5% CO₂ and 95% N₂ with the addition immediately before use of 2 mM sodium dithionite (the anoxic buffer). In the latter case, the cultures were placed in a chamber flooded with 5% CO₂ and 95% N₂. Cultures were then washed by gentle immersion in BBSS and returned to their original growth medium. These procedures were performed under aseptic conditions and with sterile solutions used throughout. Typically, this static exposure to 5 min of anoxia resulted in swollen neuronal somata with easily visible cell nuclei within 1 hr and degenerating processes and widespread neuronal death visible within 4 hr by trypan blue exclusion. Three days after the anoxia procedure, all neurons had died, leaving only glial cells on the coverslip.

Ischemia *in vivo*. The method used for ischemia induction has been described in detail previously (Mudrick and Baimbridge, 1991). Briefly, male Wistar rats weighing 300–350 gm were allowed food and water *ad libitum* until the time of surgery. After the rats were anesthetized with pentobarbital (65 mg/kg, i.p.), the carotid arteries were isolated, and a silk thread was placed around them. The femoral artery was cannulated to monitor blood pressure and to withdraw blood to lower the blood

pressure to values <40 mmHg. Both carotid arteries were then clamped for 12 min while the low blood pressure was maintained. Body temperature was monitored and kept at 36–37°C throughout the procedure. Control animals were subjected to hypotension only. After recovery, the animals were caged separately with food and water *ad libitum*.

Axotomy *in vivo*. Adult male Wistar rats weighing 300–350 gm were allowed food and water *ad libitum* until the time of surgery. Under deep pentobarbital anesthesia (65 mg/kg), the main trunk of the left facial nerve was exposed at its emergence from the stylomastoid foramen, and an ~5–8 mm section was removed. This resection of the facial nerve has been shown to induce delayed neuronal death in ~30% of the axotomized motor neurons (Angelov and Neiss, 1994). Tissue was processed in parallel to the ischemic brains.

Acute isolation of astrocytes. Transient forebrain ischemia was performed as described above. Rats were anesthetized and killed by decapitation 18–24 d after ischemia. The hippocampi were removed and kept briefly at 4°C in HCO₃⁻- and CO₂-buffered saline (in mM: NaCl 127, KCl 3, NaHCO₃ 19.5, NaH₂PO₄ 1.5, MgSO₄ 1.5, D-glucose 17.5, and CaCl₂ 1). A tissue chopper was used to cut 400 μ m sections, which were kept in HCO₃⁻- and CO₂-buffered saline until they were transferred into HCO₃⁻- and CO₂-buffered saline containing 1 mg/ml trypsin for 30 min at 37°C. The stratum radiatum of the CA1 was then microdissected and triturated in HEPES-buffered salt solution (HBSS; in mM: NaCl 139, KCl 3.5, Na₂HPO₄ 3, NaHCO₃ 2, HEPES acid 6.7, HEPES-Na 3.3, D-glucose 11, CaCl₂ 1.8, and glycine 40 μ M, pH 7.35) with 1 mg/ml trypsin inhibitor at room temperature. The cells were plated on a glass coverslip, left to adhere for 15 min, and then loaded with fluo-4 (see below). The stratum radiatum, isolated from area CA1 18–24 d after ischemia, contained mainly astrocytes, thereby decreasing the likelihood of recording from neurons (Kimelberg et al., 2000). The astrocytes were identified by morphological criteria as described previously (Köller et al., 2000) (see Fig. 6A).

Immunohistochemistry. For immunohistochemical analysis, rats were killed 24 hr–56 d after ischemia by pericardial perfusion with 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4. The brains were removed, postfixed for 1 hr at room temperature, and then stored in 30% sucrose in phosphate buffer for cryoprotection. Cryostat sections (30 μ m each) were made and stored at 4°C in 0.01 M PBS with 0.001% sodium azide. Alternate sections were taken for immunohistochemical staining, which allowed adjacent sections throughout the hippocampus to be processed for the different antibodies used. Cultures were fixed by immersion in 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4, for 1 hr at room temperature, washed in PBS, and stored at 4°C in PBS-azide.

The rabbit polyclonal antibody recognizing the intracellular C-terminal region (residues 1463–1482) of the NR2B subunit was generously provided by Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD) (Lau and Huganir, 1995) and used at a dilution of 1:1000. A polyclonal affinity-purified antibody (Chemicon, Temecula, CA) was also used at a concentration of 1:1000. A monoclonal antibody recognizing the NR1 subunit of the NMDAR (Chemicon) was used at a dilution of 1:100. A monoclonal antibody against GFAP (Roche Molecular Biochemicals, Indianapolis, IN) was used at a dilution of 1:500.

Standard immunohistochemistry was performed. Briefly, alternate tissue sections or fixed cultures on glass coverslips were rinsed in PBS, blocked, and permeabilized with 0.5% Triton X-100 and 5% BSA in PBS, and incubated overnight at 4°C in the primary antibody (NR2B, NR1, or GFAP) or the lectin GSAIB4 (20 μ g/ml). For double-labeling studies, primary antibody binding was visualized with a fluorescent secondary antibody (Alexa Fluor 488 goat anti-mouse or Alexa Fluor 594 goat anti-rabbit; Molecular Probes, Eugene, OR) at a dilution of 1:500. Sections were mounted on gelatinated slides and plated on coverslips in SlowFade Antifade (Molecular Probes). For light microscopy, we used biotinylated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or biotinylated protein A, prepared by the method of Slovicter et al. (Slovicter et al., 1991). Sections were further processed with 1:1000 diluted horseradish peroxidase–biotin–avidin complex for 1 hr followed by 0.05% DAB (Sigma, St. Louis, MO) containing 0.07% (w/v) nickel chloride and 0.01% (v/v) hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. Sections were then dehydrated and mounted under coverslips.

Antibody specificity. To check for antibody specificity, human embry-

Figure 1. Western blots probed with the NR2B antibodies obtained from Dr. R. Huganir (*A*) and Chemicon (*B*). Lanes were loaded with 25 and 50 μ g of protein obtained from transiently transfected HEK cells expressing NR1/2A, NR1/2B, or NR1/2C. Both antibodies showed high specificity for NR2B at 180 kDa, although slight cross-reactivity with NR2A could be detected with the Chemicon antibody.

onic kidney (HEK) cells were transfected with NR1 together with either NR2B, NR2A, or NR2C, as described previously (Chen et al., 1997). Cells were harvested 24–36 hr after transfection into ice-cold Eppendorf tubes in 1 ml of iced buffer containing 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 20 μ g/ml pepstatin, and 100 U/ml aprotinin in PBS (harvest buffer), sonicated for 10 sec, and centrifuged at 14,000 rpm for 30 min at 4°C. Precipitates were sonicated for 10 sec in harvest buffer containing 1% Triton X-100, mixed end-over-end for 30 min at 4°C to solubilize membrane proteins, and centrifuged (14,000 rpm for 30 min at 4°C). Supernatants were saved, and protein concentration was determined with the bicinchoninic acid protein assay (BCA kit; Pierce, Rockford, IL). Samples were stored at -80°C when not analyzed immediately. Identical quantities of protein from different membrane samples were loaded to 8% SDS-PAGE, transferred overnight to polyvinylidene difluoride membranes, probed with anti-NR2B antibodies (at dilutions of 1:1000 for the antibody from Dr. Richard Huganir or 1:200 for the Chemicon antibody), and then incubated with anti-rabbit antibody conjugated with horseradish peroxidase (diluted 1:5000; Amersham Biosciences, Arlington Heights, IL) and visualized by enhanced chemiluminescence (Amersham). Both antibodies showed high specificity for NR2B, although slight cross-reactivity with NR2A could be detected with the Chemicon antibody (Fig. 1).

Calcium imaging. Fluo-4 was purchased as the AM ester (Molecular Probes Inc.) and stored in 50 μ g aliquots at -80°C until use. Cells were loaded in a 4 μ M solution of fluo-4 AM in HBSS containing 0.05% BSA (Sigma). Postanoxic astrocytes were loaded for 1 hr, and the coverslips were then transferred to HBSS for 30 min to allow complete hydrolysis of fluo-4 AM.

Fluo-4 fluorescence was measured with a Zeiss (Oberkochen, Germany) Attofluor digital fluorescence imaging system controlled by Attofluor imaging software as described previously (Sidky and Baimbridge, 1997). A Zeiss Axiovert-10 fluorescent microscope equipped with a long-distance 40 $\times$ objective (Zeiss LD UV-Achroplan 0.6, Ph2) and a 100 W mercury arc lamp was used. Excitation light for fluo-4 was filtered through a 10 nm bandpass, 488 nm excitation filter, the position of which was determined by a computer-controlled solenoid

Figure 2. Immunohistochemistry of control rat brains showing the normal distributions of NR2B and NR1. *A, B*, Overview (scale bar, 100 μ m) of the hippocampus and high-power magnification (scale bar, 50 μ m) of the CA1 region. *A*, NR2B staining is present in the soma and dendrites of pyramidal cells throughout the hippocampus, as well as the soma and dendrites of dentate granule cells. *B*, NR1 is present in the soma and dendrites of pyramidal cells throughout the hippocampus, as well as the soma and dendrites of dentate granule cells. Glial cells from control brains showed no detectable staining with either of these antibodies.

filter changer. The filtered light was reflected by a dichroic mirror (FT-495), and the emitted light was transmitted by the dichroic mirror before being filtered by a 510 nm long-pass filter.

A coverslip containing the postanoxic astrocytes was mounted face-up in a superfusion chamber. The inflow channel was connected to a perfusion pump, whereas the outflow channel was connected to a suction line, which removed all of the superfusate present above a level of 3 mm.

Figure 3. Time course of glial NR2B expression after transient forebrain ischemia. *A–C*, Overview (scale bar, 100 μ m) of the hippocampus and high-power magnification (scale bar, 50 μ m) of the CA1 region. *A*, At 7 d after ischemia, the pyramidal cell layer (py) of the CA1 contains pyknotic neuronal cell somata, and the stratum radiatum (ra) is devoid of NR2B-positive dendrites or neurons. Numerous NR2B-positive glial cells are present within the CA1 and subicular regions but are notably absent from the CA3 region and dentate gyrus. *B*, By 28 d after ischemia, there is an increase in the number and intensity of staining of NR2B-positive glial cells throughout the CA1 and subicular regions, and little remains of pyknotic neuronal cell somata. *C*, By 42 d after ischemia, there is a marked decline in the number and intensity of staining of NR2B-positive glial cells in the CA1 and subicular regions.

Figure 4. Double labeling with glial cell markers demonstrating that, after ischemia *in vivo*, NR1 and NR2B are colocalized in astrocytes. *A*, Stratum radiatum of the CA1 region, 14 d after ischemia. Scale bar, 50 μ m. GFAP and NR2B are colocalized within astrocytes. *B*, Stratum radiatum of the CA1 21 d after ischemia. Scale bar, 50 μ m. GSAIB4 and NR2B are not colocalized, which indicates that the NR2B-immunoreactive glial cells are not microglia. *C*, Stratum radiatum of the CA1 28 d after ischemia. Scale bar, 50 μ m. NR1 and NR2B are colocalized within astrocytes, which demonstrates that both subunits necessary for functional NMDARs are present in the same cells.

Astrocytes were superfused at a rate of 2.4 ml/min at room temperature with Mg^{2+} -free HBSS.

Changes in intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) were expressed as the $\Delta F/F$ of fluo-4 fluorescence after background correction. No attempt was made to calibrate the data to absolute levels of $[Ca^{2+}]_i$, but compared with NMDA-induced changes in $[Ca^{2+}]_i$ in neurons also loaded with fluo-4, the responses in astrocytes appeared to be relatively small.

Results

Immunohistochemistry reveals novel expression of NR2B-type NMDARs in astrocytes after ischemia *in vivo*

In control animals, NR1 and NR2B staining was present throughout the hippocampus and the dentate gyrus, as described previously (Petralia et al., 1994; Charton et al., 1999) (Fig. 2). Transient forebrain ischemia resulted in selective neuronal death that was restricted to the CA1 and subicular regions of the hippocampal formation, with sparing of CA3 pyramidal cells and dentate granule cells. Starting 3 d after ischemia, both of the antibodies to NR2B detected the expression of NR2B in glial cells present in the CA1/subicular region of the dorsal hippocampus. The numbers and intensity of staining of the glial cells, seen with NR2B staining and GFAP staining (data not shown), increased over time, reaching a maximum between 14 and 28 d, and declined thereafter, with only a few immunopositive glial cells being observed after 56 d (Fig. 3). Double labeling for GFAP demonstrated clearly that NR2B-expressing glia were astrocytes (Fig. 4*A*). The pan-microglial marker GSAIB4 was used to screen for any NR2B expression on microglia, but no colocalization was found (Fig. 4*B*). The potential for functional NMDARs, which require the NR1 subunit, was confirmed by double labeling for both NR2B and NR1 subunits (Fig. 4*C*).

NR1 and NR2B are expressed in astrocytes from rat hippocampal cultures after anoxia

Postnatal hippocampal cultures that yielded neurons on a glial layer showed NR2B immunoreactivity only in the neurons and not in GFAP-immunoreactive glia (Fig. 5*A*). Subjecting the hippocampal neuronal and glial cultures to anoxia resulted in neuronal death, leaving only glial cells on the coverslip. Using immunocytochemistry, we were able to show that these remaining glial cells expressed both the NR1 and NR2B subunits and GFAP at 3 d after anoxia, thus replicating our *in vivo* findings (Fig. 5*B,C*). NR2B immunoreactivity was also seen on peripheral astrocyte processes that were not visible with staining for GFAP (Fig. 5*B*).

Astrocyte cultures (grown in the absence of neurons) were also exposed to either 5 or 60 min of anoxia with the same protocol used for neuron and glial cultures. Anoxia had no detectable effect on NR2B subunit expression as determined both immunohistochemically and by Western blot (data not shown).

Astrocytes express functional NR1 and NR2B after anoxia *in vitro* or ischemia *in vivo*

For further functional analysis of the NR2B receptor, we used the postanoxic cultures that contained NR1- and NR2B-expressing astrocytes and astrocytes isolated acutely from the CA1 of the ischemic hippocampus 18–24 d after transient forebrain ischemia. To verify that the NR1 and NR2B expressed on astrocytes *in vivo* were functional, we acutely isolated hippocampal astrocytes from the CA1 region 2–3 weeks after ischemia (Fig. 6*A*), a time point at which our immunohistochemical studies had shown high levels of expression of both NR1 and NR2B subunits in the CA1 and subicular regions. The astrocytes were loaded with fluo-4, and exposure to 0.5–1 mM NMDA resulted in a monophasic increase in $[Ca^{2+}]_i$ (Fig. 6*B*). A concentration–response experiment established that 0.5–1 mM NMDA was necessary in the acutely isolated astrocytes to elicit an increase in $[Ca^{2+}]_i$ measurable in the whole cells (Fig. 6*C*). Astrocytes acutely isolated from the hippocampus of adult control animals (subjected only to hypotension, not to ischemia) showed no response to NMDA, whereas they responded to glutamate with increases in $[Ca^{2+}]_i$ (Fig. 6*D*). Application of equimolar amounts of the competitive NMDAR antagonist APV and NMDA (0.5–1 mM) did not result in any increases in $[Ca^{2+}]_i$, whereas a subsequent superfusion of the same astrocyte with NMDA alone elicited an increase in $[Ca^{2+}]_i$ (Fig. 6*E*). To pharmacologically establish the presence of the NR2B subtype, cells were stimulated with 1 mM NMDA and 3 μ M ifenprodil, a selective blocker of NR1 and NR2B, and then with 1 mM NMDA alone. The astrocytes showed only an attenuated response to NMDA with ifenprodil but were responsive to NMDA alone (Fig. 6*F*).

For analysis of the postanoxic astrocytes *in vitro*, coverslips with hippocampal cultures were taken at 3 d after anoxia for calcium imaging studies. To ensure the specificity of the re-

sponse, in some experiments cells were superfused for 2 min with a blocking mixture that contained 60 μM L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) to block the glutamate transporter and thereby inhibit reversal of the transporter (Anderson et al., 2001), 40 μM 6-cyano-7-nitroquinoxaline-2,3-dione to block AMPA receptors, and 500 nM tetrodotoxin to block sodium channels. In the absence of neurons in these postanoxic neuron and glial cultures, this blocking mixture was intended to prevent any non-NMDA signaling by glutamate that may have been released from the astrocytes themselves in response to NMDA. In these experiments, the astrocytes were then stimulated with 1 mM NMDA in the blocking mixture for 5 min and allowed to recover. This resulted in an oscillatory increase in $[\text{Ca}^{2+}]_i$ that began at the cell process and rapidly propagated to the soma (Fig. 7). This Ca^{2+} response to NMDA could be reversibly blocked with APV, and similar responses to NMDA were found in the absence of the blocking cocktail (data not shown). Although PDC applied acutely rather than by a prolonged incubation could release glutamate from neurons by heteroexchange of PDC for glutamate and by impairing glutamate uptake (Anderson et al., 2001), the fact that we saw no calcium response to the blocking mixture alone supports our morphological data indicating complete loss of pyramidal neurons from the hippocampal CA1 region.

Axotomy does not induce NR2B expression on astrocytes

Axotomy induces delayed neuronal death of $\sim 30\%$ of all facial motor neurons. It is a well established model to study astrogliosis in the vicinity of both dying and surviving neurons. It was thus chosen as a model to investigate the influence of neuronal death to glial NMDAR expression. None of the reactive astrocytes in the nucleus of the axotomized facial nerve showed any NR2B immunoreactivity 14 d after surgery (Fig. 8).

Discussion

We have shown that ischemia *in vivo* and anoxia *in vitro* result in expression within astrocytes of the NR2B subunit of the NMDAR that is colocalized in both instances with the NR1 subunit required for functional NMDARs. In the ischemia model, the presence of astrocytes that expressed NMDARs was restricted to the CA1 and subicular regions in which significant neuronal loss occurred. Furthermore, NR2B subunit expression was first observed 3 d after ischemia, reached a peak between 14 and 28 d, but then declined such that by 56 d, only a few NR2B-expressing astrocytes were still present. Direct evidence for functional NR1 and NR2B was obtained from calcium imaging of astrocytes isolated acutely from the hippocampus after ischemia or from astrocytes that remained in postnatal hippocampal cultures 3 d after anoxia-induced neuronal death. Our data therefore extend previous observations of the presence of NMDARs in astrocytes after ischemia (Gottlieb and Matute, 1997) in that we demonstrate the

Figure 5. Double labeling with glial cell markers demonstrating that, after anoxia *in vitro*, NR1 and NR2B are colocalized in astrocytes. *A*, In a control postnatal hippocampal culture after 10 d *in vitro*, NR2B immunoreactivity is restricted to neuronal cells, and GFAP immunoreactivity is restricted to glial cells, with no overlap. Scale bar, 20 μm . *B*, In a postnatal hippocampal culture 3 d after anoxia, GFAP and NR2B are colocalized in the surviving astrocytes. Note that the NR2B immunoreactivity extends into distal glial processes that are not visible with staining for GFAP. Scale bar, 50 μm . *C*, In a postnatal hippocampal culture 3 d after anoxia, NR1 and NR2B are colocalized in astrocytes, which demonstrates that both subunits necessary for functional NMDARs are present in the same cells. Scale bar, 20 μm .

involvement of the NR2B subunits and show that they form functional NMDARs in association with the NR1 subunit.

The expression of NMDARs in astrocytes could be an intrinsic response to anoxia and ischemia or a response related to the accompanying neuronal death. The former would appear unlikely, because NMDA expression in pure astrocyte cultures was not influenced by a 5 or even 60 min exposure to anoxia. It would appear more likely that neuronal death provides a trigger for the generation of reactive astrocytes and that part of their response is the expression of NMDARs. However, in this context, it is interesting to note that we did not detect NMDARs in the astrocytes present in the facial motor nucleus ipsilateral to facial nerve axotomy, a well established model of glial activation and apoptotic neuronal death. This suggests that our observations may represent either a specific response of glial cells in the CA1/subicular region of the hippocampal formation or that they are related more to the type of early necrotic neuronal death seen, for example, in our *in vitro* model of anoxia rather than the delayed apoptotic neuronal death seen after axotomy. Additional experiments are needed to resolve these possibilities.

Our evidence for functional NMDAR expression in reactive astrocytes is based on their intracellular calcium response to NMDA. The dose of NMDA required to induce a calcium response was high, in the 0.5–1 mM range. The EC_{50} for NMDA-evoked peak currents recorded from murine cortical and diencephalic neurons is $\sim 60 \mu\text{M}$ (Sather et al., 1992). Although the lack of calcium responses at NMDA concentrations $< 500 \mu\text{M}$ in the present calcium imaging experiments on astrocytes cannot be

Figure 6. Functional NMDARs in astrocytes isolated acutely from the CA1 region of the hippocampus 20 d after ischemia. *A*, Phase-contrast image of acutely isolated astrocytes. *B*, Superfusion with 1 mM NMDA (solid bar) for 1 min resulted in a monophasic increase in $[Ca^{2+}]_i$, represented as the $\Delta F/F$ of fluo-4 fluorescence after background correction. *C*, Concentration–response experiment showing the need for high concentrations of NMDA to elicit a calcium response. *D*, An astrocyte isolated acutely from a nonischemic hippocampus showed no response to 5 min of NMDA, whereas superfusion of glutamate (glu) resulted in an increase in $[Ca^{2+}]_i$. *E*, Superfusion of 1 mM NMDA and 1 mM APV for 5 min elicited no change in $[Ca^{2+}]_i$, whereas superfusion of 1 mM NMDA alone resulted in a monophasic increase in $[Ca^{2+}]_i$. *F*, Superfusion with 3 μ M ifenprodil alone (1) or 3 μ M ifenprodil with 1 mM NMDA (2) elicited only an attenuated calcium response. After washing with HBSS (3), a calcium response could be elicited with 1 mM NMDA alone (4). The representative examples shown in *B–F* were typical of results found in two to three astrocytes.

compared directly with electrophysiological results using neuronal preparations, there are unique features of astrocytes that might explain our results. First, the density of surface NMDARs expressed in astrocytes may be low compared with neurons. Second, calcium compartmentalization, buffering, or both may be different between astrocytes and neurons so that a larger calcium influx is required to induce a measurable change in free cytosolic calcium. Alternately, we cannot rule out the coexpression of NR2C or NR2D, along with NR2B and NR1, in astrocytes after ischemia as an explanation for the low sensitivity of the NMDARs in the present calcium imaging studies, because when coexpressed along with NR1 subunits in HEK-293 cells, the EC_{50} values for glutamate-induced increases in calcium are substantially greater for NR2C or NR2D than for NR2A or NR2B (Grant et al., 1998).

The functional implications of glutamate receptors expressed in glia remain speculative (Gallo and Russell, 1995; Kimelberg, 1995; Steinhäuser and Gallo, 1996) and have been reviewed previously (Gallo and Ghiani, 2000). They may play a role in development, or they may increase glial ion homeostatic functions and the capacity of glia to take up glutamate in response to injury. It has been suggested that NMDARs on astrocytes may be important in a glutamate-mediated signal for glial proliferation (Uchi-

hori and Puro, 1993), whereas NMDA stimulation of neurons appears to have the opposite effect, inhibiting neurogenesis in the dentate gyrus (Cameron et al., 1998).

In our experiments, NMDAR-induced calcium responses were present in astrocytes only after anoxia or ischemia. In astrocytes from postanoxic neuron and glial cultures, increases in $[Ca^{2+}]_i$ were initiated in the processes and progressed to the soma, which suggests that the NMDARs were located predominantly on the surface of astrocyte processes. This observation is in accordance with data from a recent report that also demonstrated a calcium elevation located predominantly in astrocyte processes (Schipke et al., 2001). This conclusion was not evident from the immunohistochemical data, but the methods used would have detected both surface and intracellular NMDAR subunits. Studies without permeabilization of the cell, which could have selected for surface receptors, were not possible, because the NR2B antibody used binds to the intracellular C terminus of the receptor. In contrast, astrocytes isolated acutely from the ischemic hippocampus had NMDAR-induced calcium responses that were monophasic. One difference between the preparations used that may account for the differences in their NMDAR-induced calcium responses is that during isolation of astrocytes from the ischemic hippocampus, their processes are sheared off. In addition, the nature of the calcium responses may be influenced by the fact that cell–cell contacts were not present in the acutely isolated

cells but were present in astrocytes from postanoxic neuron and glial cultures

In contrast to previous reports, we were not able to demonstrate the presence of NMDAR expression by immunohistochemistry, nor could we elicit an NMDA-induced calcium response in rat astrocytes that had not been subjected to anoxia *in vitro* or ischemia *in vivo*. This might be attributable to species differences, differences in the type of preparation, or both. For example, Schipke et al. (2001) found NMDA-induced increases in calcium in astrocytes in acute slices prepared from mouse cortex, whereas in the present study, we used cultured or acutely isolated astrocytes from rat hippocampus. In the latter preparation, our inability to elicit a calcium response in control animals after NMDA stimulation could also be related to the shearing off of the processes. A much higher receptor density in addition to the presence of functional receptors on the soma in the postischemic astrocytes could be an explanation for the response seen there. A previous study (Kondoh et al., 2001) found NMDAR currents in human astrocytes isolated from the white matter surrounding metastatic brain tumor tissue, but although the study was able to rule out that the astrocytes studied were tumor cells, they still might have been reactive astrocytes (expressing NMDARs)

Figure 7. NMDA-induced increase in $[Ca^{2+}]_i$ demonstrates the presence of functional NMDARs in postanoxic astrocytes in culture. The representative example of a postnatal hippocampal astrocyte, 3 d after anoxia, shows a polyphasic increase in $[Ca^{2+}]_i$ resulting from the superfusion of 1 mM NMDA for 10 min (indicated by the solid line). The dotted line indicates the superfusion of a medium containing a blocking mixture to eliminate the contribution of non-NMDA glutamate receptors (see Results). A similar result was found in a total of four astrocytes examined. $[Ca^{2+}]_i$ is represented as the $\Delta F/F$ of fluo-4 fluorescence after background correction.

surrounding the tumor. Other studies showed NMDA-evoked responses in specialized glial populations, such as Mueller cells in the retina or Bergmann glia in the cerebellum (Muller et al., 1993; Luque and Richards, 1995; López et al., 1997).

Our model of transient forebrain ischemia results in the selective loss of CA1 pyramidal neurons, whereas the CA3 pyramidal cells remain intact. Similarly, reactive astrocytes are located in the CA1 but are absent from the CA3 regions. It is therefore of interest to note that a number of recent reports have demonstrated differences between the astroglial cells in these two regions. For example, dye-coupling experiments revealed large syncytia of astrocytes extending throughout the different layers of the CA1 region, whereas the syncytia in CA3 were significantly smaller and never extended into different layers (D'Ambrosio et al., 1998). Also, the electrophysiological properties were different, with CA1 astrocytes showing predominantly a linear I – V relation, whereas CA3 astrocytes exhibited a complex or inwardly rectifying I – V relation. In-

wardly rectifying profiles are better-suited for buffering of K^+ , because they allow K^+ influx but impede K^+ efflux. CA3 spatial buffering is further facilitated by an extracellular volume fraction in stratum pyramidale that has been estimated to be 50% greater than that in CA1 (McBain et al., 1990). Other aspects of the heterogeneity of the astrocyte population have been reviewed recently (Walz, 2000).

Glial cells have numerous receptors and ion channels that enable them to maintain a complex Ca^{2+} -signaling machinery. Depending on the morphology of the glial cells and the distribution of Ca^{2+} channels and receptors on their surface, the nature of the Ca^{2+} signal can vary significantly. Increases in $[Ca^{2+}]_i$ are thought either to result from Ca^{2+} influx from the extracellular space, mediated, for example, by AMPA receptors (which lack the GluR2 subunit), voltage-operated Ca^{2+} channels, or both, or to be related to release from intracellular stores after activation of metabotropic glutamate receptors (e.g., mGluR5). It has been shown that Ca^{2+} signals can spread through the cell and, via gap junctions, through the glial syncytium (Cornell-Bell et al., 1990; Verkhratsky et al., 1998).

The potential consequences of Ca^{2+} signaling in glial cells range from the stimulation of nitric oxide synthase to the activation of second-messenger systems that are linked to gene expression (including those for cytoskeletal elements) and effects on other ion channels, such as an increased activation of Ca^{2+} -dependent K^+ channels (Quandt and MacVicar, 1986; Barres et al., 1990). This activation could then increase K^+ buffering and siphoning capacity in astrocytes, contributing to extracellular ion homeostasis. It has also been suggested that a Ca^{2+} elevation in glial cells may result in the release of glutamate in amounts sufficient to stimulate adjacent neurons (Parpura and Haydon, 2000). This glutamate release could be of importance for stimulating presynaptic glutamate receptors on Schaffer collaterals, thus ensuring that these axons do not degenerate because of lack of a target (Arabadzisz and Freund, 1999).

In summary, we have demonstrated for the first time the expression of functional NMDARs in astrocytes of the ischemic hippocampus of the rat and in cultured hippocampal astrocytes after anoxia. An understanding of the role of astrocytes that express functional NR2B-subtype NMDARs in response to ischemic and anoxic injury may give insight into the remodeling of an injured brain region and guide development of novel therapies after stroke.

Figure 8. *A*, GFAP staining of the ipsilateral and contralateral facial nucleus 14 d after unilateral resection of the facial nerve. Reactive astrocytes can only be detected in the ipsilateral nucleus. *B*, NR2B staining of the ipsilateral and contralateral facial nucleus of the same animal. No NR2B immunoreactivity can be found on neurons or glia.

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