Diazepam, Given Postischemia, Protects Selectively Vulnerable Neurons in the Rat Hippocampus and Striatum

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Following cerebral ischemia, certain populations of neurons degenerate. Excessive accumulation of excitatory amino acids in the synaptic cleft, activation of excitatory amino acid receptors, and influx of calcium into neurons play a key role in the development of ischemia-induced neuronal death. We hypothesized that neuroprotection may be achieved by enhancing inhibitory (i.e., γ -aminobutyric acid, GABA) neurotransmission to offset excitation. Diazepam, a drug that increases GABA-induced chloride channel opening, was administered (10 mg/kg, i.p.) to rats 1 and 2 hr following 15 min of transient global ischemia, when hippocampal GABA levels, increased during ischemia, returned to basal. Rats were maintained normothermic during ischemia and became hypothermic following the injections of diazepam. Four days later, rats were sacrificed and the brains were examined for neuronal degeneration and the presence of GABA_A receptors labeled by ³⁵S-t-butylbicyclophosphorothionate (³⁵S-TBPS). There was substantial neuroprotection of striatal neurons and pyramidal neurons in the CA1 area of the hippocampus. In addition, diazepam prevented the loss of ³⁵S-TBPS binding sites in the striatum and in the dendritic fields of the CA1 hippocampus following ischemia. Since hypothermia, itself, is neuroprotective, we determined if hypothermia was required for the ability of diazepam to produce neuroprotection. Diazepam was microinjected into the CA1 hippocampus 1 and 2 hr following ischemia, and rats remained normothermic. Four days later, diazepam still produced substantial protection of hippocampal neurons. Thus, postischemic hypothermia may have contributed to the neuroprotection by diazepam when it was administered systemically, but the neuroprotective effect of diazepam did not require hypothermia. We conclude that delayed enhancement of GABAergic neurotransmission directly at the site of vulnerability following an ischemic event protects the vulnerable neurons from death.

[Key words: cerebral ischemia, neuronal death, GABA_A receptor, diazepam, neuroprotection, hypothermia]

Transient cerebral ischemia is produced when the brain is deprived temporarily of oxygen and glucose. After cardiac arrest with resuscitation or cardiopulmonary bypass surgery, cerebral ischemia can lead to problems with cognition and memory, to serious neurological problems such as sensorimotor deficits and seizures, and to death (Levy et al., 1985; Volpe and Petito, 1985; Newburger et al., 1993). In humans and in animals subjected to transient cerebral ischemia, specific neurons degenerate following the ischemic episode (Ito et al., 1975; Kirino, 1982; Pulsinelli et al., 1982; Petito et al., 1987). The striatum, hippocampus, and somatosensory cortex are particularly sensitive. In animal models, neurons in the striatum and somatosensory cortex degenerate quickly after the onset of reperfusion (within 1 d), while pyramidal neurons in the CA1 sector of the hippocampus degenerate 3–4 d later (“delayed neuronal death”) (Kirino, 1982; Pulsinelli et al., 1982; Crain et al., 1988). The delayed degeneration of neurons provides the opportunity for neuroprotective agents to be administered following the ischemic insult.

There have been numerous studies in animals to assess neuroprotective efficacy of various classes of drugs following transient global (cerebral) and focal ischemia, although there is no satisfactory treatment for humans. Most of these studies have focused on glutamate receptor antagonists because excessive activation of glutamate receptors by excitatory amino acid neurotransmitters released during ischemia and the subsequent rise in intracellular calcium are important triggers in the development of ischemia-induced neuronal death (Rothman, 1984; Benveniste et al., 1984; Choi, 1988; Schurr and Rigor, 1992). Neuroprotective efficacy (limited to complete) has been demonstrated not only by glutamate receptor antagonists (Simon et al., 1984; Gill et al., 1988; Sheardown et al., 1990; Nellgard and Wieloch, 1992; Li and Buchan, 1993), but also by drugs affecting several other neurotransmitter systems, by calcium channel antagonists, and by lipid peroxidation inhibitors (for review, Kriegstein, 1990; Schurr and Rigor, 1992).

We have been interested in the role of inhibitory neurotransmission in ischemia-induced neuronal death. Inhibitory neurotransmission [mediated principally by γ -aminobutyric acid (GABA)] normally provides a balance for excitatory neurotransmission (mediated principally by glutamate) in many areas of the brain. Thus, pharmacological enhancement of GABA_A neurotransmission is an alternative therapeutic strategy to the use of excitatory amino acid antagonists for neuroprotection following an ischemic event. The benzodiazepines, which have anxiolytic, sedative/hypnotic, anticonvulsant, and muscle relaxant properties, are the most commonly used drugs to enhance GABA_A neurotransmission (Tallman et al., 1980; Rall, 1990).

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Figure 1. Ischemia induces accumulation of GABA in the CA1 area of the hippocampus. Extracellular GABA concentrations in the CA1 hippocampus were measured using *in vivo* microdialysis before, during, and after transient global ischemia as described in Materials and Methods. Ischemia was initiated at time 0. Each curve is from an individual rat. Values are GABA (μM) in the dialysate.

Benzodiazepines, such as diazepam, bind to a site associated with the GABA_A receptor to increase the frequency of opening of the GABA-gated chloride channel (Study and Barker, 1981; Rall, 1990). Benzodiazepines and other drugs that enhance GABA neurotransmission (e.g., GABA agonists and uptake and metabolism inhibitors) produce varying degrees of neuroprotection when administered before or during cerebral ischemia in rodents (Sternau et al., 1989; Kato et al., 1990; Johansen and Diemer, 1991; Shuaib et al., 1992; Shuaib et al., 1993). Based on these studies, GABA-enhancing drugs should be efficacious when used before and during surgical procedures which limit blood flow to the brain. Given immediately after cerebral ischemia in gerbils, benzodiazepines have failed to produce neuroprotection (Sternau et al., 1989). However, there may be a "therapeutic window" after an ischemic event has already occurred, during which a drug can be administered and produce neuroprotection. Therefore, we have determined if the delayed administration of the benzodiazepine, diazepam, following transient global ischemia is neuroprotective in selectively vulnerable regions of the rat striatum and hippocampus. In addition, we have determined if neuroprotection by diazepam results from enhancement of GABA neurotransmission directly at the site of vulnerability.

These results have been presented in preliminary form (Yu et al., 1993) and support our recent study using a gerbil model of transient forebrain ischemia (Schwartz et al., 1994a).

Materials and Methods

Surgical procedures. Adult male Wistar rats (300–350 gm) were subjected to the 4 vessel occlusion technique, as described previously by

Benveniste et al. (1984), with modifications. The vertebral arteries of rats were electrocauterized (under halothane anesthesia) and the following day the carotid arteries were occluded for 15 min. Rats were anesthetized with 2.5% halothane in breathing air, and the tail artery was cannulated so that blood pressure could be monitored and blood could be withdrawn as necessary. Subdermal cortical EEG leads were placed over the cerebral cortex for the monitoring of EEG, and temperature probes were placed in the temporalis muscle and rectum. After rats were stabilized, the carotid arteries were isolated and occluded with silk sutures for 15 min. Blood pressure was reduced to approximately 60 mm Hg by withdrawal of blood through the tail artery cannula. Both temporalis muscle and rectal temperatures were monitored throughout the procedure, and maintained between 36 and 37°C using a heating pad and an incandescent lamp placed over the head. EEG activity became isoelectric within seconds of the occlusion and the halothane anesthesia was discontinued. After 15 min, reflow was established by release of the sutures and verified visually. Any blood withdrawn was returned to the rat via the tail artery. Sham-operated rats received identical treatment, except the carotid arteries were not occluded. Rats recovered after surgery in separate cages.

In a separate experiment, rats were implanted with guide cannulas 1 week prior to microinjections into the CA1 sector of the hippocampus on both sides of the brain. The same time course for dosing was used as for the systemic dosing regimen. Sixty and 120 minutes following ischemia, diazepam (50 ng or 1.25 μg) was microinjected directly into the CA1 sector of the hippocampus at the rate of 0.2 $\mu\text{l}/\text{min}$ over a 2.5 min period. The injection cannula was positioned so that it did not pierce the hippocampus; the tip was aimed at the alveus. The coordinates for injection were: 3.3 mm posterior to bregma, 2.2 mm lateral to bregma, angled 10° from vertical, and 2.42 mm below the surface of the skull. The contralateral hippocampus was injected with vehicle (20% polyethylene glycol in artificial cerebrospinal fluid). Rats were maintained normothermic during the ischemia, and rectal temperature was measured for 3 hr following the ischemia and injections of diazepam.

In vivo microdialysis. A separate group of rats was used for *in vivo* microdialysis recordings. Two hours prior to the occlusion of the carotid arteries, a microdialysis probe (CMA-12, 1 mm tip; Bio Analytical Systems, West Lafayette, IN) was implanted stereotactically into the CA1 area of the right dorsal hippocampus. The coordinates were: 3.6 mm posterior to bregma, 2.0 mm lateral to bregma, and 3.5 mm below the skull surface. The hippocampus was perfused with artificial cerebrospinal fluid consisting of 122 mM NaCl, 3 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgSO_4 , 0.4 mM KH_2PO_4 , and 25 mM NaHCO_3 , pH 7.35, at a rate of 2 $\mu\text{l}/\text{min}$. Occlusion of the carotid arteries commenced after 2 hr of perfusion to obtain a stable baseline. Perfusates (30 μl) were collected every 15 min and stored at -20°C until assayed for amino acid concentrations by HPLC.

Measurement of extracellular GABA. GABA and other amino acids, including glutamate, were measured in the dialysates after derivatization with *o*-phthalaldehyde (OPA) by HPLC with dual detection, as previously reported, with modifications (Schwartz et al., 1994b). Dialysates (30 μl) were mixed with homoserine as the internal standard and incubated with OPA and β -mercaptoethanol for 2.5 min at 6°C. Twenty microliters of the reaction mixture was injected onto an ESA HR-80 C 18 column (3 μm). The mobile phase was isocratic and contained 0.1 M sodium phosphate dibasic buffer in 25% methanol (pH = 6.75). The flow rate was 1.2 ml/min. For the electrochemical detector, the guard cell potential was set at 650 mV, and the analytical cell potentials were set at -400 and $+600$ mV. The fluorescence detector was set to an excitation wavelength of 330 nm, and the emission filter was 418 nm. Chromatograms were analyzed on an LDC LCTalk workstation (Riviera Beach, FL), which calculated the ratio of sample peak area to the internal standard peak area. The sensitivity for GABA detection was 50 fmol (with 2:1 signal to noise).

Drug injections. Ischemic and sham-operated rats were injected i.p. with either 10 mg/kg diazepam or vehicle (33% polyethylene glycol in saline) 60 min and again 120 min following the onset of ischemia. Rectal temperature was monitored for several hours to assess the degree of hypothermia induced by diazepam. Microinjections of diazepam into the hippocampus are described under Surgical procedures.

Histological assessment of neuronal degeneration. Four days following onset of reperfusion, rats were decapitated, and the brains were removed, frozen in isopentane, and stored at -70°C . Frozen brains were cut into 20 μm sections at the level of the striatum and the dorsal hippocampus. Adjacent sections were mounted on subbed glass slides for histological

Figure 2. Diazepam produces hypothermia following cerebral ischemia. Rectal temperatures were measured in rats subjected to global cerebral ischemia for 15 min and administered vehicle or diazepam (DZ) (10 mg/kg, i.p.) 60 and 120 min following ischemia. Data are the mean $\pm$ SEM for 6–7 rats. $\circ$, sham; $\blacklozenge$, ischemia; $\square$, sham + DZ; $\blacksquare$, ischemia + DZ.

analysis and ^{35}S -t-butylbicyclophosphorothionate (^{35}S -TBPS) autoradiography (see below). Slices were stained with thionin and viable cells were visualized using a light microscope. To quantitate damage to the striatum, the area of damage was digitized using Image 1.49 image analysis software and compared to the total striatal area to give the percent of area damaged. In the hippocampus, the extent of damage was measured in 1 mm segments of the CA1a, CA1b, and CA1c areas of dorsal hippocampus using a variation of the rating scale described

by Pulsinelli et al. (1982). Histological examination of bilateral hippocampi was made by two blinded observers using the following rating scale: 0—<25% cells lost, 1—25–50% cells lost, 2—50–75% cells lost, and 3—>75% cells lost.

In a separate experiment, when diazepam was microinjected into the hippocampus, rats were sacrificed 4 d after the ischemia by transcardial perfusion with saline then paraformaldehyde (4%). The perfused brains were sliced into 40 μ m sections using a freezing microtome and subjected

Figure 3. Diazepam protects striatal neurons and spares GABA_A receptors in dorsolateral striatum following cerebral ischemia. Rats were sacrificed 4 d following transient global ischemia, and thionin-stained sections of striatum were examined for degeneration as described in Materials and Methods. Rats were administered diazepam (10 mg/kg) 60 and 120 min after cerebral ischemia. Each symbol is from an individual rat. The horizontal lines represent the mean value for each group. There was significant protection of striatal neurons by diazepam. $p = 0.0005$, Kruskal–Wallis test followed by the Mann–Whitney U test ($n = 6, 6, 11$, and 13 , respectively).

Figure 4. Representative sections from thionin stained striatum and from ³⁵S-TBPS autoradiograms show complete neuroprotection by diazepam. Rats were sacrificed 4 d following the onset of reperfusion. Adjacent striatal sections were stained with thionin for histological analysis and digitized (left) or subjected to ³⁵S-TBPS autoradiography to measure the density of GABA_A receptors (right). *A* = sham, *B* = sham + diazepam, *C* = ischemia, *D* = ischemia + diazepam. Arrows point to area of damage and loss of binding. In the autoradiograms, the greatest density of ³⁵S-TBPS binding is indicated in black.

Figure 5. Diazepam protects hippocampal neurons and spares GABA_A receptors in dendritic fields of CA1 hippocampus following cerebral ischemia. Rats were sacrificed 4 d following transient global ischemia, and thionin-stained sections of hippocampus were examined for degeneration as described in Materials and Methods. Rats were administered diazepam (10 mg/kg) 60 and 120 min after cerebral ischemia. Each symbol represents an individual rat. Diazepam produced significant protection of pyramidal neurons in the CA1 area of the hippocampus. $p = 0.002$, Kruskal-Wallis test followed by the Mann-Whitney U test ($n = 6, 6, 10$, and 12 , respectively).

to silver impregnation stain as described previously (Crain et al., 1988; Miles and Schwartz, 1991). Degeneration of hippocampal pyramidal cells was measured semiquantitatively by comparing the digitized area of the CA1 pyramidal cell layer showing argyrophilic soma to the total area of the CA1 pyramidal cell layer (Crain et al., 1988; Miles and Schwartz, 1991).

Autoradiography of ^{35}S -TBPS binding to GABA_A receptors. ^{35}S -TBPS binding to GABA-gated chloride channels was assessed in adjacent sections to those used for histology as we described previously (Edgar and Schwartz, 1990). Slide-mounted sections for autoradiography were desiccated overnight at 4°C and stored at -70°C . Sections were thawed and preincubated at room temperature in buffer containing 50 mM $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 200 mM NaCl, pH 7.4, and 1 mM EDTA to remove endogenous GABA. Sections were incubated for 3 hr at room temperature in buffer described above (without EDTA) containing 1 to 2 nM ^{35}S -TBPS (New England Nuclear, Boston, MA; specific activity 50–100 Ci/mmol). Following incubation, sections were washed two times for 15 min in buffer, dipped in distilled water, and dried under a stream of air. Non-specific binding, determined in the presence of excess picrotoxin (100 μM) in the incubation medium, was less than 5% of total binding and undetectable on film (Edgar and Schwartz, 1990). Sections were apposed to Hyperfilm-Betamax (Amersham, Arlington Heights IL) in x-ray cassettes for 2–3 d. Film was processed using Kodak D-19 developer and fixer. Autoradiographic images were digitized and quantitated using Image 1.49 software from NIH (Bethesda, MD) and with a MAC Quadra 950. Optical densities were converted to fmol using a standard curve generated with plastic strips of ^{14}C standards calibrated to ^{35}S standards prepared from brain-mash sections as described previously (Edgar and Schwartz, 1990; Miller, 1991).

Data analysis. Differences among group means were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test for individual comparisons. Paired comparisons were made using the paired Student's t test. A probability level of <0.05 was considered to be statistically significant.

Results

The first experiment was carried out to determine the best time to inject diazepam following transient cerebral ischemia. During

cerebral ischemia, extracellular GABA levels become elevated substantially (Globus et al., 1988). When GABA levels are saturating, diazepam will not have an effect because it shifts the GABA dose-response curve to the left in a parallel manner (Tallman et al., 1980; Study and Barker, 1981). Therefore, we used *in vivo* microdialysis to monitor the extracellular levels of GABA during and after cerebral ischemia. Extracellular GABA was elevated 4–50 times above the basal level in response to the ischemia and returned toward basal levels by 45–60 min following ischemia (Fig. 1). Based on these findings, we injected diazepam 60 min following ischemia in all subsequent experiments.

During transient global ischemia, normothermia was maintained (rectal temperatures ranged from 36.5 – 37.5°C) (Fig. 2). Temporalis muscle temperature was maintained between 36.0 – 37.0°C during ischemia. One hour following the onset of ischemia, rats were injected with a sedating dose of diazepam (10 mg/kg, i.p.). This dose of diazepam produced hypothermia; diazepam decreased rectal temperature by 1.5°C over the next hour (Fig. 2). A second injection of diazepam 2 hr after ischemia decreased rectal temperature by an additional 1 – 1.5°C over the next 2 hr. The hypothermia reached a plateau at 33.5°C by 4 hr following the ischemia.

Four days following reperfusion, rats were sacrificed, and sections of striatum and hippocampus were examined for neuronal loss. Delayed administration of diazepam produced substantial protection of striatal neurons (Figs. 3, 4). Protection of striatal neurons was nearly complete to complete in 69% of the rats; little to no protection was observed in 31% of the rats. Complete protection of striatal sections stained with thionin from individual rats is shown in Figure 4. Diazepam also produced significant protection of pyramidal neurons in the CA1 sector of

Figure 6. Representative sections from thionin-stained hippocampus and from ^{35}S -TBPS autoradiograms show complete neuroprotection by diazepam. Rats were sacrificed 4 d following the onset of reperfusion. Adjacent sections at the level of the dorsal hippocampus were stained with thionin for histological analysis (*left*) or subjected to ^{35}S -TBPS autoradiography to measure the density of GABA $_A$ receptors (*right*). *A* = sham, *B* = sham + diazepam, *C* = ischemia, *D* = ischemia + diazepam. Black pointers indicate the CA1 sector in which there is loss of pyramidal cell bodies and ^{35}S -TBPS binding in dendritic fields.

Table 1. Binding of ^{35}S -TBPS to GABA-gated chloride channels 4 d following the onset of reperfusion

Brain region	^{35}S -TBPS bound (fmol/mg protein)			
	Sham	Sham + diazepam	Ischemia	Ischemia + diazepam
Striatum	14.6 $\pm$ 1.9	10.4 $\pm$ 1.3	3.7 $\pm$ 0.4*	9.8 $\pm$ 1.1
Hippocampus				
CA1 stratum oriens	28.4 $\pm$ 1.7	31.1 $\pm$ 3.3	13.3 $\pm$ 1.9*	21.6 $\pm$ 3.8
CA1 stratum radiatum	35.7 $\pm$ 2.9	33.7 $\pm$ 6.7	16.0 $\pm$ 2.5*	25.6 $\pm$ 4.7
CA1 lac. moleculare	56.3 $\pm$ 4.3	63.8 $\pm$ 6.1	29.7 $\pm$ 5.9*	45.3 $\pm$ 7.8
CA3 stratum oriens	23.9 $\pm$ 2.7	23.1 $\pm$ 3.1	13.7 $\pm$ 2.0	15.4 $\pm$ 2.0
Dentate gyrus				
Stratum moleculare	34.3 $\pm$ 3.8	39.5 $\pm$ 5.3	25.2 $\pm$ 6.1	32.4 $\pm$ 4.1
Thalamus				
VPL nucleus	21.7 $\pm$ 2.8	26.3 $\pm$ 3.1	19.0 $\pm$ 1.9	21.7 $\pm$ 3.3
VPM nucleus	37.4 $\pm$ 3.9	40.5 $\pm$ 5.6	29.8 $\pm$ 1.5	35.2 $\pm$ 5.4

Brain sections adjacent to those used for histology were subjected to ^{35}S -TBPS autoradiography as described in Materials and Methods. Data are femtomoles of ^{35}S -TBPS bound/mg protein $\pm$ the SEM of 5–7 rats. Abbreviations: VPL, ventroposterior lateral; VPM, ventroposterior medial.

* $p < 0.05$, compared to sham; all other comparisons are not significantly different, Kruskal–Wallis test followed by the Mann–Whitney U test.

the hippocampus (Figs. 5, 6). Protection was complete in 33% of the rats. Partial protection was obtained in 33%, and no protection was obtained in 33% of the rats. Complete protection of hippocampal sections from individual rats is shown in Figure 6.

Previously, we reported that GABA_A receptors labeled with ^{35}S -TBPS were lost concomitantly with degeneration of striatal and hippocampal neurons following transient cerebral ischemia (Miles et al., 1992). To determine if GABA_A receptors were spared along with protected neurons, we measured the binding of ^{35}S -TBPS to GABA-gated chloride channels in brain sections adjacent to those used for histological analysis (Figs. 4 and 6 and Table 1). ^{35}S -TBPS binding was reduced significantly in the dorsolateral striatum and in the dendritic fields (strata oriens,

radiatum, and lacunosum moleculare) of the CA1 sector of the hippocampus (Table 1). ^{35}S -TBPS binding was not affected in areas of the thalamus that were also subjected to ischemia. Diazepam significantly prevented the loss of GABA_A receptors in the dorsolateral striatum and in the dendritic fields of the hippocampus (Table 1). In brain sections from individual rats, the complete sparing of GABA_A receptors by diazepam can be observed (Figs. 4, 6).

Hypothermia, produced by diazepam, is a common effect of many neuroprotective drugs. Hypothermia, induced physically, has also been shown to be neuroprotective in animals subjected to cerebral ischemia (Busto et al., 1987; Minamisawa et al., 1990; Welsh et al., 1990). To determine if the neuroprotective efficacy of diazepam was related to its ability to produce hy-

Figure 7. Diazepam, injected into the CA1 hippocampus, fails to produce hypothermia. Sixty and 120 minutes following ischemia, rats were microinjected with vehicle into the CA1 sector of the hippocampus on one side of the brain and with diazepam (1.25 μg) on the contralateral side. Rectal temperature was monitored before, during, and after drug injections. Rats remained normothermic throughout the experiment. Data are the mean $\pm$ SEM of eight rats. Error bars are omitted when smaller than the symbol.

Figure 8. Diazepam, injected into the CA1 hippocampus after cerebral ischemia, is neuroprotective. Sixty and 120 minutes following ischemia, rats were microinjected with vehicle into the CA1 sector of the hippocampus on one side of the brain and with diazepam (1.25 μ g) on the contralateral side. The extent of neuronal degeneration in the CA1 sector of the hippocampus was assessed as described in Materials and Methods. Data are the mean $\pm$ SEM from six rats. Diazepam produced significant protection of pyramidal cells compared to the contralateral side ($p = 0.002$, Student's paired t test).

pothemia, we maintained normothermia (physically) after the injection of diazepam. However, the physical manipulation of core temperature was very stressful to the animal (unpublished observations) so we considered this to be an unacceptable experiment. Instead, we injected diazepam directly into the hippocampus, 60 and 120 min after the ischemia (the same dosing regimen used for systemic administration). When diazepam was administered directly into the hippocampus, no hypothermia was produced (Fig. 7). Four days after the onset of perfusion, the hippocampus on each side of the brain was examined for neuronal degeneration. Compared to the vehicle-injected side, diazepam produced substantial protection of pyramidal neurons in the CA1 sector on the contralateral side (Fig. 8). Complete neuroprotection of the entire hippocampus was achieved even in a severely ischemic rat (Fig. 9*A,B*). This was surprising, since it is almost impossible to achieve neuroprotection pharmacologically when the ischemic insult is severe (Buchan et al., 1991; Warner et al., 1991). Under higher magnification, individual degenerating CA1 pyramidal cells showing argyrophilia from a mildly ischemic rat were observed along with complete neuroprotection on the contralateral side injected with diazepam (Fig. 9*C,D*).

Discussion

We have shown that delayed administration of diazepam after cerebral ischemia prevents neuronal degeneration in vulnerable regions of the striatum and hippocampus. Postischemic hypothermia may have contributed to the neuroprotection by diazepam when it was administered systemically but the neuroprotective effect of diazepam did not require hypothermia (when administered into the hippocampus). Furthermore, a repeated dosing regimen was not required. The dosing regimen (two injections commencing 1 hr after ischemia) produced excellent neuroprotective efficacy in the striatum (69% of the rats were completely protected). The greater efficacy of diazepam (for a given dose) in the striatum compared to hippocampus is similar

to the greater efficacy of both glutamate receptor antagonists and hypothermia to protect striatal neurons compared to hippocampal neurons (Warner et al., 1991). In the hippocampus, a greater degree of neuroprotection might have been achieved with a higher dose or with an additional dose of diazepam. However, we have found that this dose of diazepam (given 30 min after ischemia) produces neuroprotection to a greater degree in the gerbil hippocampus, compared to the rat (Schwartz et al., 1994a). The neuroprotective efficacy of diazepam was evident 4 d after the ischemic episode. Additional experiments will be required to determine if the neuroprotection is long lasting or whether damage is merely delayed. Interestingly, Lyden and Lonzo (1994) have found that the combination of a GABA agonist (muscimol) and an *N*-methyl-D-aspartate antagonist (MK-801) is neuroprotective in the rat cortex, when assessed 3 months after an embolic stroke.

The time after ischemia at which diazepam is administered may be critical (the "therapeutic window"). During and immediately following cerebral ischemia, GABA is released from neurons and accumulates in the extracellular space due to failure of energy-dependent reuptake pumps (Globus et al., 1988; Nakata et al., 1993). Benzodiazepines enhance GABA neurotransmission by shifting the GABA concentration-response curve to the left, thereby increasing GABA potency (Tallman et al., 1980; Study and Barker, 1981). Therefore, benzodiazepines should only be efficacious when extracellular GABA concentrations have declined back to basal levels, i.e., by 30–45 min following ischemia (see Fig. 1). This may explain why diazepam failed to protect hippocampal neurons when it was given 5 min after transient forebrain ischemia in the gerbil (Sternau et al., 1989). It is not known if diazepam administered 5 min after ischemia in the rat is neuroprotective. In another study, a much lower dose of diazepam, administered postischemia, did not protect hippocampal neurons, yet it did protect cerebral cortical neurons (Voll and Auer, 1991).

The neuroprotection achieved by diazepam injected directly into the hippocampus raises two important issues. First, the experiment shows that neuroprotection occurs in the absence of hypothermia. There is considerable evidence that hypothermia, present during an ischemic event, is neuroprotective (Busto et al., 1987; Welsh et al., 1990). However, hypothermia, induced after ischemia, during the first 2 hr of reperfusion, is neuroprotective in some cases but not others (Busto et al., 1989; Welsh and Harris, 1991; Coimbra and Wieloch, 1994). Thus, while hypothermia is not required for neuroprotection by diazepam, still it may be involved in the protection achieved when diazepam is administered systemically. This is highly desirable, since hypothermia is induced routinely as a neuroprotective measure during cardiopulmonary bypass surgery and cardiac arrest, or after stroke and brain injury (Newburger et al., 1993). Second, the experiment demonstrates for the first time that enhancement of GABA neurotransmission within the area of vulnerability after an ischemic event is neuroprotective. This provides indirect evidence that GABA neurotransmission plays a role in the development of neuronal death following an ischemic insult. Direct evidence has been difficult to show, but several studies, when considered together, support this theory. First, the functional responses of GABA_A receptors, *in vitro*, are attenuated by several mediators of ischemia-induced neuronal death. These include activation of phospholipase A₂, generation of arachidonic acid and its metabolites, generation of superoxide radicals (Schwartz et al., 1988; Schwartz and Yu, 1992), and the influx

VEHICLE

DIAZEPAM

Figure 9. Diazepam, injected into the CA1 hippocampus, completely protects hippocampal neurons in severely and mildly ischemic rats. Sections of hippocampus were subjected to silver degeneration staining to indicate the extent of damage to hippocampal neurons 4 d following 15 min of transient global ischemia. *A* and *B*, Hippocampi from a single animal showing severe ischemia. Vehicle was injected into the CA1 area of the hippocampus on one side of the brain (*panel A*) and diazepam ($1.25 \mu\text{g}$) was injected into the contralateral hippocampus 60 and 120 min following ischemia (*panel B*). There was complete protection of the entire hippocampus on the side injected with diazepam; the *dark areas* represent degeneration of axon terminals whose cell bodies originate in the unprotected contralateral hippocampus. Scale bar in *A* = $250 \mu\text{m}$. *C* and *D*, Higher power magnification of the pyramidal cell body layer in hippocampi from a mildly ischemic rat. Argyrophilic neurons showing degeneration are observed in the hippocampus injected with vehicle (*C*). Complete protection of the contralateral side was achieved with the microinjection of diazepam (50 ng) (*D*). Scale bar in *C* = $30 \mu\text{m}$.

of calcium into neurons (Inoue et al., 1986; Schwartz et al., 1994b). Second, we have shown that there is a rapid decline (within hours) in GABA_A receptor subunit mRNA levels in hippocampal neurons well before they die after an ischemic event (Li et al., 1993). Third, GABA_A responses decline in forebrain synaptoneuroosomes from gerbils sacrificed within 2 hr of the ischemic episode (Verheul et al., 1993). Fourth, increased neuronal excitability or a loss of synaptic inhibition (presumably mediated by GABA) can occur early in the reperfusion phase (Suzuki et al., 1983; Kawai et al., 1992). Thus, after cerebral ischemia, inhibitory neurotransmission may be attenuated, thereby contributing to or promoting neuronal death. The neuroprotective efficacy of GABA-enhancing agents at a critical time following an ischemic event supports this idea.

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