

## Midazolam Improves Electrophysiologic Recovery after Anoxia and Reduces the Changes in ATP Levels and Calcium Influx during Anoxia in the Rat Hippocampal Slice

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Since blockers of excitatory transmission have been shown to reduce anoxic and ischemic neuronal damage, augmentation of inhibitory transmission by agents such as midazolam might have a similar protective effect. Rat hippocampal slices were maintained *in vitro* and used to determine whether and by what mechanism midazolam improves recovery of evoked responses after anoxia. The Schaffer collateral pathway in the slice was stimulated electrically, and an extracellular potential, the evoked population spike, was recorded from the CA1 pyramidal cells, which are postsynaptic. The slices were made anoxic by substituting artificial cerebrospinal fluid aerated with 95% nitrogen-5% carbon dioxide for fluid aerated with 95% oxygen-5% carbon dioxide. Percentage recovery was expressed as the amplitude of the evoked population spike 60 min after anoxia divided by its preanoxic amplitude. Protection in this model is defined as a significant ( $P < 0.05$ ) improvement in percentage recovery compared to the recovery of untreated slices. There was no recovery of the response recorded from CA1 pyramidal cells after 5 min of anoxia ( $4 \pm 2\%$ ) (mean  $\pm$  standard error of the mean [SEM]). Slices were treated with midazolam 10 min before, during, and 10 min after anoxia. Midazolam ( $1 \mu\text{M}$ ) did not enhance recovery after anoxia when dissolved either in water ( $3 \pm 3\%$ ) or in dimethyl sulfoxide (DMSO) ( $1 \pm 1\%$ ). A higher concentration of midazolam ( $100 \mu\text{M}$ ) did enhance recovery when dissolved in DMSO ( $27 \pm 7\%$ ) but not when dissolved in water ( $5 \pm 2\%$ ). To test whether prolonged pretreatment with midazolam dissolved in water would enhance recovery, slices were treated for 30 min prior to anoxia. Under these conditions,  $100 \mu\text{M}$  midazolam dissolved in water significantly improved recovery ( $63 \pm 13\%$ ). Flumazenil ( $33 \mu\text{M}$ ), a central benzodiazepine antagonist, completely blocked this protective effect of midazolam ( $5 \pm 4\%$ ). Surprisingly, high concentrations of midazolam ( $100 \mu\text{M}$ ) increased the size of the response before anoxia ( $132 \pm 7\%$ ). This effect was not reversed by flumazenil ( $33 \mu\text{M}$ ) and therefore is believed not to be due to activation of the central benzodiazepine receptor. Adenosine triphosphate (ATP) was maintained at a significantly higher level during anoxia when  $100 \mu\text{M}$  midazolam was present in the bathing medium ( $1.58 \pm 0.12$  vs.  $2.02 \pm 0.13$  nm/mg dry weight). Net calcium influx, as measured by  $^{45}\text{Ca}$  uptake, was reduced during anoxia with high concentrations of midazolam ( $6.56 \pm 0.18$  vs.  $4.9 \pm 0.13$  nm/mg dry weight). The authors conclude that high concentrations of midazolam protect against anoxic damage

to the hippocampus in this *in vitro* model. This protection may be due to reduction of calcium influx and maintenance of ATP levels by midazolam during anoxia. (Key words: Anesthetics, hypnotics: midazolam. Antagonists, benzodiazepines: flumazenil. Brain, hippocampal slice: ischemia; anoxia; ATP; calcium.)

RECENT STUDIES have demonstrated that blockers of receptors for the excitatory amino acid transmitter glutamate may reduce the extent of anoxic and ischemic damage to neurons.<sup>1-3</sup> Moreover, it is believed that decreasing neuronal metabolism prior to an anoxic insult enhances the resistance of neurons to oxygen deprivation.<sup>4</sup> We therefore reasoned that augmenting inhibitory transmission might have a similar effect to reducing excitation. Benzodiazepines are believed to exert their pharmacologic effects through the  $\gamma$ -aminobutyric acid (GABA<sub>A</sub>) receptor, enhancing the inhibitory action of GABA.<sup>5</sup> This mechanism of action should decrease neuronal transmission and metabolism. Other drugs, such as barbiturates, which may work, in part, through separate recognition sites on the same GABA<sub>A</sub> receptor<sup>6</sup> have been shown to be beneficial if administered prior to ischemia.<sup>7-9</sup>

We decided to investigate the mechanism of midazolam's protection against anoxic damage because this water-soluble benzodiazepine is commonly used in anesthetic practice and has many theoretical advantages for patients with central nervous system (CNS) damage undergoing surgery or requiring intensive care.<sup>10-11</sup> The recent introduction of flumazenil, a specific competitive antagonist of the benzodiazepine receptor, makes the sedation reversible.<sup>12</sup>

Our study examines the ability of midazolam to reduce damage caused by anoxia to the CA1 pyramidal cells of the rat hippocampus *in vitro*. The hippocampal slice was used as a model system since it allows a comparison of an agent's ability to enhance recovery of electrophysiologic responses of an identified population of neurons with the agent's biochemical effects on these neurons during anoxia.<sup>13</sup> The ability of flumazenil to block the action of midazolam on electrophysiologic recovery from anoxia is also examined. Reduced adenosine triphosphate (ATP) levels and increased calcium influx during anoxia are believed to be important factors promoting anoxic damage.<sup>14,15</sup> We therefore measured intracellular ATP levels and calcium influx during anoxia and compared the effect of midazolam on these parameters with its electrophysiologic effect.

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## Materials and Methods

The experiments reported in this article were approved by the Animal Care and Use Committee at the State University of New York Health Science Center at Brooklyn. The methods have been described in detail elsewhere.<sup>13-16</sup> Male Sprague-Dawley rats, 110-120 days old, were decapitated and in each the hippocampus was rapidly dissected from the brain, sliced transverse to the long axis in 500- $\mu$ m sections, and placed on nylon mesh held to a Plexiglas grid. The slices were deeply submerged (1 cm below the air-fluid interface) and superfused with artificial cerebrospinal fluid (aCSF) at a rate of 60 ml/min. The aCSF was equilibrated with 95% oxygen-5% carbon dioxide and the temperature maintained at 37° C. The composition of the aCSF was (millimolar): NaCl 126, KCl 3, KH<sub>2</sub>PO<sub>4</sub> 1.4, MgSO<sub>4</sub> 1.3, CaCl<sub>2</sub> 1.4, NaHCO<sub>3</sub> 26, and glucose 4; pH was 7.4. The conditions for maintaining the slice *in vitro* were chosen to simulate, as closely as possible, those found *in situ*. The aeration of the aCSF with 95% oxygen is required to assure adequate oxygen for neurons in the middle of the slice, which are 250  $\mu$ m from the surface of the tissue. This concentration of oxygen is used almost universally in brain slice preparations.

One hour after the dissection, a bipolar stimulating electrode and a monopolar metal recording electrode were placed in the slice. To record the population spike evoked from the CA1 pyramidal cells, the stimulating electrode was placed in the Schaffer collateral pathway, which monosynaptically activates these cells. The recording electrode was placed in the CA1 pyramidal cell layer to record the summed action potentials of many pyramidal cells that are activated simultaneously. The size of the population spike is proportional to the number of neurons generating action potentials. A detailed description and diagram of this pathway has been provided by Andersen *et al.*<sup>17</sup> The stimulating pulses were biphasic (each phase 100  $\mu$ s), and the voltage was adjusted to provide a maximal response at the beginning of the experiments; parameters were not adjusted afterward. The slice was stimulated once every 10 s throughout the experiment. Responses were monitored for 1 h to ensure stability of the preparation. Only slices that had a stable response were used for the experiment. If slices are not subjected to anoxia, they demonstrate stable responses for 6 h.<sup>16</sup>

Anoxia was generated by superfusing the slice with aCSF preequilibrated with 95% nitrogen-5% carbon dioxide. The CA1 pyramidal cells were exposed to 5 min of anoxia; there was little recovery of the evoked response after this period of anoxia in untreated slices. The population spike was measured as the mean amplitude, in microvolts, of the negative and positive arms of the population spike. The recovery of neuronal transmission was expressed as the amplitude of the evoked population spike after 60 min of normoxic reperfusion after anoxia, divided

by its preanoxic, predrug amplitude. Midazolam maleate (1  $\mu$ M or 100  $\mu$ M) and/or dimethyl sulfoxide (0.5%) (DMSO) was added to the aCSF 10 min before anoxia, was present during anoxia, and was washed out 10 min after the anoxic insult. The lower concentration of midazolam was chosen since this concentration activates the central benzodiazepine receptor. We also examined 100  $\mu$ M midazolam because this concentration of benzodiazepine has been shown to have additional effects on calcium influx.<sup>18,19</sup>

In order to determine if DMSO was required for midazolam to exert its protective effect or if DMSO only accelerated the access of the drug to an active site, we tested the effect of a 30-min pretreatment with midazolam in the absence of DMSO. This protocol increased the duration of the period after slice preparation until anoxia from 2 h to 2 h and 20 min and the period of electrical stimulation from 60 to 80 min. In order to exclude the possibility that the prolongation of these periods might have a protective effect independent of the effect of midazolam, we repeated the experiments without any drugs, extending the periods before anoxia.

After anoxia, for all groups, the slices were reperfused with oxygenated aCSF for 60 min; drugs were washed from the slices 10 min after anoxia. In our preparation, the maximum recovery usually is present after 30 min of reperfusion with oxygenated aCSF, and no additional recovery is observed for the next 3 h.<sup>16</sup> It is not possible to follow recovery much longer in this *in vitro* preparation. Midazolam was dissolved either in DMSO (final concentration 0.5%) or water. Flumazenil, an antagonist of the benzodiazepine receptor, was dissolved in 0.5% DMSO when used. All control experiments were performed either with no drugs or with 0.5% DMSO in aCSF. The significance ( $P < 0.05$ ) of the recovery from anoxia was determined using analysis of variance (ANOVA) and Scheffe's tests.

In the analysis of the change in population spike amplitude independent of anoxia and due to the various drugs, the responses from the same slice before and after drug application but prior to anoxia were compared and the percent change due to the drug calculated. This change was analyzed for a number of experiments using the same drug administration protocol with Student's *t* test.

The time until complete block of the population spike during anoxia was measured for each group. In the first series of experiments (table 1), the effect of two concentrations of midazolam with and without DMSO were compared to untreated controls. These data were analyzed with an ANOVA and the Student-Newman-Keuls procedure for planned multiple comparisons. In a second series of experiments (table 2), midazolam's effect on time to signal loss was compared to untreated controls with Student's *t* test. In addition, Student's *t* test was used to

TABLE 1. Recovery of the Population Spike after 5 min of Anoxia and Time until Complete Response Block During Anoxia

	n	% Recovery	Time (s)
Untreated	24	4 ± 2	101 ± 3
DMSO (0.5%)	5	2 ± 1	110 ± 4
Midazolam (1 μM) in H <sub>2</sub> O	6	3 ± 3	108 ± 7
Midazolam (1 μM) in DMSO	5	1 ± 1	100 ± 3
Midazolam (100 μM) in H <sub>2</sub> O	6	5 ± 2	133 ± 8*
Midazolam (100 μM) in DMSO	5	27 ± 7†	127 ± 4*

Drug treatment began 10 minutes before anoxia, present during and 10 min after anoxia.

\* Significantly different from the untreated group.

† Significantly different from all other groups.

compare independently the flumazenil-midazolam combination with midazolam alone in order to test flumazenil's ability to antagonize midazolam's effect.

Biochemical parameters were measured in slices treated similarly to those in the electrophysiologic experiments. The tissue was prepared and placed on Plexiglas grids as described above. Each grid was then placed in a small beaker with aCSF and bubbled with 95% oxygen-5% carbon dioxide. To generate anoxia, the aCSF was bubbled with 95% nitrogen-5% carbon dioxide.

For the experiments in which ATP was measured, midazolam (100 μM) dissolved in 0.5% DMSO was added to the aCSF 10 min before anoxia and remained in the solution until the completion of the experiment. At the end of the anoxic period the slices were frozen rapidly in liquid nitrogen and lyophilized. CA1 regions were dissected from the slices and weighed, and the ATP was extracted.<sup>20</sup> ATP was measured using the luciferin-luciferase assay.<sup>21</sup>

The calcium influx studies were carried out in a similar manner except that radioactive <sup>45</sup>Ca was added at the beginning of anoxia. In these experiments, midazolam was dissolved in water and was added to the aCSF 30 min before anoxia. At the end of anoxia the slices were removed to 3-4°C, modified aCSF (2 mM lanthanum chloride and no phosphate or bicarbonate) for 60 min. This step allowed the washout of extracellular <sup>45</sup>Ca while preserving intracellular levels, since lanthanum inhibits calcium efflux.<sup>15</sup> The slices then were frozen, lyophilized, dissected, and weighed, and finally were assayed in a liquid scintillation counter.

In all the biochemical studies significance was determined with an ANOVA and Student's *t* test. All values are expressed as mean ± SEM.

## Results

### RECOVERY OF THE EVOKED POPULATION SPIKE AFTER ANOXIA

The population spike recorded from untreated CA1 pyramidal cells does not recover after 5 min of anoxia.

Sixty minutes after the end of anoxia, the amplitude of the evoked population spike reaches 4% of its preanoxic level (table 1).

When 1 μM or 100 μM midazolam was dissolved in water and applied 10 min before anoxia, neither concentration had an effect on the recovery of the population spike amplitude after anoxia (table 1).

To ensure that midazolam was reaching its site of action, we dissolved the drug in DMSO, a commonly used vehicle for drug delivery. DMSO (0.5%) alone did not affect the recovery after anoxia (table 1). The lower concentration of midazolam (1 μM) dissolved in DMSO also did not improve recovery from anoxia; however, 100 μM midazolam did provide protection against anoxic damage when dissolved in DMSO (table 1). The population spike recovered to 27% of its preanoxic, predrug amplitude; this was significantly greater than the recovery with no drugs, with DMSO alone, or with midazolam dissolved in water added 10 min prior to anoxia (table 1). Thus, DMSO enhanced the protective effect of midazolam when both were present 10 min prior to anoxia.

Slices also were treated with midazolam for an extended, 30-min period to ensure that midazolam dissolved in water had sufficient time to exert its effect. These experiments necessitated a longer preincubation time before anoxia, and therefore the following controls were required. In the absence of any drugs, the amplitude of the CA1 evoked population spike remained stable (106 ± 3%) 2 h and 20 min after slice preparation as compared to the amplitude after 2 h (102 ± 4%). There was no recovery of untreated slices from 5 min of anoxia after the prolonged preanoxic period (9%) (table 2).

When 100 μM midazolam in water was added to aCSF 30 min before anoxia, the evoked population spike demonstrated significant improvement of recovery after anoxia (63%) (table 2). An example of a single experiment demonstrating this enhanced recovery is shown in figure 1. This example also shows the effect of midazolam on the response before anoxia, which is examined in detail later in this paper.

Flumazenil (33 μM in 0.5% DMSO), a benzodiazepine antagonist, did not affect recovery from anoxia (4%) when

TABLE 2. Recovery of the Population Spike after 5 min of Anoxia and Time Until Complete Response Block During Anoxia

	n	% Recovery	Time (s)
Untreated	13	9 ± 5	116 ± 5
Midazolam (100 μM) in H <sub>2</sub> O	7	63 ± 13†	147 ± 17*
Flumazenil (33 μM) in DMSO	5	4 ± 4	117 ± 7
Flumazenil (33 μM) in DMSO and midazolam (100 μM) in H <sub>2</sub> O	5	5 ± 4	131 ± 14

Drug treatment began 30 minutes before anoxia and was present during and 10 min after anoxia.

\* Significantly different from the untreated group.

† Significantly different from all other groups.

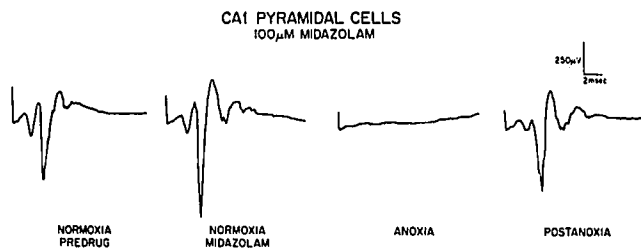


FIG. 1. The effect of midazolam on the recovery of responses evoked from CA1 pyramidal cells after 5 min of anoxia. Responses are recorded from the CA1 pyramidal cell layer after stimulation of the Schaffer collaterals. The first smaller spike on the trace is caused by action potentials in the Schaffer collaterals which are presynaptic to the pyramidal cell neurons. The second and larger spike, on which all of our experiments are based, is due to action potentials in the CA1 pyramidal cell neurons. Representative traces are shown, before anoxia, before anoxia with 100  $\mu\text{M}$  midazolam, during anoxia and 60 min after anoxia. Midazolam, dissolved in water, is added to the aCSF 30 min before the beginning of anoxia and washed out 10 min after the end of the anoxic period. Midazolam increased the size of the population spike from CA1 pyramidal cells before anoxia and allowed better recovery of this response after anoxia.

added alone (table 2). The enhanced recovery after anoxia observed with 100  $\mu\text{M}$  midazolam alone was not seen when 100  $\mu\text{M}$  midazolam and 33  $\mu\text{M}$  flumazenil were added simultaneously (5%) (table 2). An example of this for a single experiment is shown in figure 2.

#### RESPONSE AMPLITUDE BEFORE AND DURING ANOXIA

A high concentration of midazolam (100  $\mu\text{M}$ ) increased the size of the population spike before anoxia (figure 1). The mean increases in the groups demonstrating a significant increase were 132, 141, and 122% (table 3). The only group in which 100  $\mu\text{M}$  midazolam did not significantly increase the size of the response was the group in which midazolam was present for 30 min; however, even this group showed a trend toward increased amplitude (mean 121%).

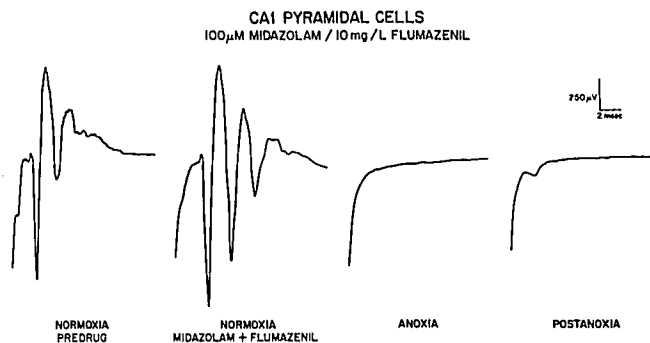


FIG. 2. The effect of midazolam in combination with flumazenil on recovery of responses evoked from CA1 pyramidal cells after 5 min of anoxia. The presynaptic response is difficult to see in these traces. The first large response is the population spike due to action potentials in the CA1 pyramidal cells. Representative traces are shown, before anoxia, before anoxia with 100  $\mu\text{M}$  midazolam and 33  $\mu\text{M}$  flumazenil, during anoxia, and 60 min after anoxia. Midazolam dissolved in water and flumazenil dissolved in DMSO are added simultaneously 30 min before anoxia and washed out 10 min after the end of the anoxic period. The size of the population spike increased in the preanoxic period when both midazolam and flumazenil were present. There is no recovery from anoxia in the presence of both of these drugs.

Flumazenil alone caused a small (14%) but significant decrease in the response 10 min after its application (table 3). This effect was transitory and was not seen when this drug had been present for 30 min. When slices were treated simultaneously with flumazenil and midazolam, the former did not block the effect of the latter to increase the size of the population spike. Quite the opposite, there was a nonsignificant trend for flumazenil to increase further the size of the response. Thus, flumazenil blocked the protective effect of midazolam on anoxic damage but did not reverse midazolam's ability to increase the amplitude of the population spike before anoxia.

The population spike was reduced and eventually completely blocked during anoxia. In the first series of experiments, where short drug preincubation times were

TABLE 3. Percent Change in Population Spike Amplitude before Anoxia

	n	10 min	n	30 min
Untreated: 60 versus 50 min	24	101 $\pm$ 2		
Untreated: 60 versus 50 min and 80 versus 50 min	13	102 $\pm$ 4	11	106 $\pm$ 3
DMSO (0.5%)	5	104 $\pm$ 3		
Midazolam (1 $\mu\text{M}$ ) in H <sub>2</sub> O	6	100 $\pm$ 5		
Midazolam (1 $\mu\text{M}$ ) in DMSO	5	116 $\pm$ 6		
Midazolam (100 $\mu\text{M}$ ) in H <sub>2</sub> O	6	132 $\pm$ 7*		
Midazolam (100 $\mu\text{M}$ ) in DMSO	5	141 $\pm$ 8*		
Midazolam (100 $\mu\text{M}$ ) in H <sub>2</sub> O	7	122 $\pm$ 8†	7	121 $\pm$ 9
Flumazenil (33 $\mu\text{M}$ ) in DMSO	5	86 $\pm$ 2*	5	98 $\pm$ 2
Midazolam (100 $\mu\text{M}$ ), flumazenil (33 $\mu\text{M}$ )	5	138 $\pm$ 13†	5	139 $\pm$ 12†

Amplitude at 50 minutes of stimulation = 100%.

\*  $P < 0.01$ .

†  $P < 0.05$ .

used (table 1), the two groups with 100  $\mu\text{M}$  midazolam showed significant prolongation of the time until signal block compared to untreated controls. Neither low concentrations of midazolam (1  $\mu\text{M}$ ) nor DMSO alone affected this time. In untreated tissue from the second series of experiments (table 2), the response is blocked after  $116 \pm 5$  s. Midazolam (100  $\mu\text{M}$ ) significantly prolonged the time required for the signal to disappear ( $147 \pm 17$  s). When midazolam and flumazenil were applied simultaneously, the time for signal disappearance was  $131 \pm 14$  s; this value was not significantly different from that for the midazolam-treated tissue. Flumazenil alone had no significant effect ( $117 \pm 7$  s) compared to untreated tissue.

#### ATP AND CALCIUM AT THE END OF ANOXIA

The effect of midazolam on ATP levels after 5 min of anoxia was measured. During anoxia in untreated slices, ATP decreased to 33% of its level before anoxia (table 4). DMSO alone did not sustain ATP levels during anoxia. In slices treated 10 min prior to anoxia with 100  $\mu\text{M}$  midazolam dissolved in DMSO, ATP levels did not decrease as much as in untreated slices. ATP levels decreased to 42% of their value before anoxia. Thus, midazolam significantly improved ATP levels during anoxia.

High intracellular calcium levels have been implicated in anoxic damage; therefore, the effect of midazolam on calcium influx during anoxia was measured. During 5 min of anoxia, net calcium influx increased to 113% of the level found during 5 min of normal oxygen content (table 5). Midazolam (100  $\mu\text{M}$ ) significantly reduced the net calcium influx during 5 min of anoxia to 84% of the level found with normal oxygen. Thus, midazolam treatment before and during anoxia not only reduced calcium uptake during anoxia; it also reduced the uptake to levels below that found during normal oxygen conditions.

#### Discussion

Results of this study demonstrate that in our model, high concentrations of midazolam, if dissolved in DMSO or if present for a prolonged period of time before 5 min of anoxia, can protect against the irreversible loss of the postsynaptic population spike in CA1 pyramidal cells. The

TABLE 4. Effect of 5 min of Anoxia on ATP Levels in the CA1 Region of the Hippocampal Slice

	n	ATP (nM/mg dry tissue)
Untreated, normoxia	25	$4.80 \pm 0.11$
Untreated, anoxia	24	$1.58 \pm 0.12$
DMSO (0.5%), anoxia	20	$1.33 \pm 0.11$
Midazolam (100 $\mu\text{M}$ ) in DMSO, anoxia	24	$2.02 \pm 0.13^*$

ATP values are mean  $\pm$  SEM.

\* Significantly different from untreated, anoxia  $P < 0.05$ .

TABLE 5. Effect of 5 min of Anoxia on Calcium Influx in the CA1 Region of the Hippocampal Slice

	n	Calcium (nM/mg dry tissue)
Untreated, normoxia	20	$5.82 \pm 0.11$
Untreated, anoxia	19	$6.56 \pm 0.18^*$
Midazolam (1 $\mu\text{M}$ ), anoxia	22	$6.39 \pm 0.16$
Midazolam (100 $\mu\text{M}$ ), anoxia	18	$4.9 \pm 0.13^{*\dagger}$

Calcium values are mean  $\pm$  SEM.

\* Significantly different from normoxia,  $P < 0.001$ .

† Significantly different from anoxia,  $P < 0.001$ .

degree of protection found with midazolam is similar to that found with blockers of the N-methyl-D-aspartate receptor<sup>2</sup> and 10  $\mu\text{M}$  magnesium.<sup>22</sup> Midazolam increased the size of the population spike in the preanoxic period; this was somewhat surprising, since benzodiazepines potentiate the inhibitory effects of GABA.<sup>5</sup> The mechanism by which midazolam exerts its protective effect was investigated using biochemical techniques that measure the levels of ATP and calcium influx in tissue from the CA1 region of the hippocampus. It was found that tissue pretreated with midazolam maintained higher ATP levels during anoxia than did untreated tissue and that calcium influx during anoxia was reduced in the midazolam-treated tissue.

The amplitude of the population spike is proportional to the number of neurons firing action potentials. A 5-min anoxic insult to the CA1 cells results in an irreversible decrease of the size of the response, despite reoxygenation. This effect is due to either permanent neuronal damage or to a permanent block of transmission at the synapse. The end result is that only a few surviving neurons fire in response to a stimulus, and the evoked response is either very small or is absent altogether. Midazolam increases the recovery of the population spike amplitude after anoxia most probably by reducing the effect of anoxia on neuronal homeostasis and/or on synaptic transmission.

The loss of the population spike during anoxia is believed to be due to the inhibition of the postsynaptic cells<sup>23</sup> and the loss of energy-supply dependent sodium and potassium transmembrane concentration gradients,<sup>24</sup> both of which would make the cells, for a time at least, reversibly unexcitable. These changes are not a direct cause of the lack of signal recovery after anoxia, since upon prompt reoxygenation the ionic gradients and inhibition of transmission return to preanoxic values.<sup>14</sup> Midazolam 100  $\mu\text{M}$  significantly prolonged the time to blockade of the evoked response during anoxia; this was followed by improved recovery in only two groups of experiments. However, in all groups that recovered there was a prolongation of the time to response block. These findings indicate that the observed effect is not sufficient but may

be necessary to enhance recovery of the evoked population spike after an anoxic insult. The attenuated decrease in ATP and the reduced calcium entry seen with midazolam may contribute to the increased time to response block during anoxia by better preserving the sodium-potassium pump activity and by decreasing inhibition through a decrease in the calcium-activated potassium conductance.<sup>23</sup>

Two clear and distinct effects were found with midazolam. One was an increase in the size of the population spike in the preanoxic period, and the other was protection against anoxic damage. These effects could be mediated through a specific benzodiazepine receptor, or they could reflect a nonspecific drug action. A high-affinity benzodiazepine binding site has been well characterized and is referred to as the central benzodiazepine receptor due to its distribution in the CNS.<sup>25</sup> Binding to this site on the GABA-benzodiazepine receptor complex potentiates the effects of GABA, an inhibitory neurotransmitter. In our model, GABA reduces the amplitude of the evoked population spike before anoxia.<sup>26</sup> The effect of midazolam is opposite that of GABA and may be due to an action not mediated through the GABA-benzodiazepine receptor complex. This has been supported by preliminary experiments that did not find any protection with GABA, even at doses that completely blocked the population spike.<sup>26</sup> Additionally, flumazenil, an antagonist of the central benzodiazepine receptor,<sup>27,28</sup> did not reverse the increase in size of the preanoxic response due to exposure to midazolam, further supporting the possibility that this effect is not a consequence of the activation of the central benzodiazepine receptor.

The increase in the population spike amplitude may be due to the activation of a separate and distinct type of benzodiazepine receptor that is found on mitochondria<sup>29</sup> and that is not coupled with a GABA recognition site and not antagonized by flumazenil.<sup>27</sup> The highly specific mitochondrial benzodiazepine agonist, Ro5-4864, is proconvulsant<sup>30,31</sup>; thus, an enhanced excitability mediated through the mitochondrial benzodiazepine receptors is consistent with the increase in the population spike amplitude before anoxia. Moreover, 1  $\mu\text{M}$  midazolam does not affect population spike amplitude even though it should activate the central benzodiazepine receptors. Another explanation of the increased population spike with 100  $\mu\text{M}$  midazolam is that the blockade of calcium entry by midazolam reduces the calcium-activated potassium conductance. This potassium current inhibits neurons by hyperpolarizing them; therefore, reducing this current would make the neurons more excitable.

Protection against anoxic damage was seen with 100  $\mu\text{M}$  midazolam and was reversed by flumazenil, a highly specific antagonist of the central benzodiazepine receptor.<sup>27,28</sup> It thus appears that midazolam protects through

activation of this receptor. Arguing against this is the observation that 1  $\mu\text{M}$  midazolam does not protect and does not affect anoxic calcium influx. Low concentrations (1  $\mu\text{M}$  or lower) of midazolam<sup>28,32</sup> or other benzodiazepines<sup>6,12</sup> are sufficient to activate the central benzodiazepine receptors. In explanation of this discrepancy, it is possible that there is a unique action of high concentrations of midazolam at the benzodiazepine receptor, an action that is responsible for the observed protective effect and that is reversed by flumazenil. Alternatively, mitochondrial receptor activation, occurring at high midazolam concentration, simultaneous with the activation of the central benzodiazepine receptor, may be required for protection. Antagonism of the central benzodiazepine receptor component hypothetically would abolish the protective effect. A nonspecific mechanism of protection independent of either of these receptors is also possible.

Damage to the GABA transmission system could be responsible for the lack of protective effect with 1  $\mu\text{M}$  midazolam in our preparation. The preparation has been tested for inhibition damage by paired pulse stimulation, a technique commonly used to measure recurrent inhibition in the hippocampus. In preliminary experiments, we found a mean inhibition of 58% when the pulses were delivered at an interpulse interval of 15 ms. This agrees well with data from the hippocampus *in vivo*,<sup>33</sup> suggesting that at least recurrent inhibition in our preparation is not seriously altered.

We did not measure the effect of 1  $\mu\text{M}$  midazolam on inhibition in our preparation; however, other studies examining CA1 pyramidal cells in the hippocampal slice found an enhancement of inhibition and no effect on excitation with this concentration.<sup>28</sup> Thus, the lack of a reduction in population spike amplitude with 1  $\mu\text{M}$  midazolam in our preparation is not an indication of an inability of midazolam to effect inhibition.

Tissue pretreated for 10 min with 100  $\mu\text{M}$  midazolam dissolved in DMSO was protected against damage, while pretreatment for 10 min with midazolam (100  $\mu\text{M}$ ) dissolved in water was not. A 30-min pretreatment with midazolam (100  $\mu\text{M}$ ) dissolved in water was required in order to demonstrate recovery after anoxia. It is difficult to explain these results since midazolam is water-soluble. One possible explanation is that for protection to occur the drug must interact with an intracellular site, possibly the mitochondrial benzodiazepine receptor, and that DMSO facilitates transport of the drug across the cell membrane. Another possibility is that midazolam acts only extracellularly but that its access to neurons in the slice is slow. A mechanism that might explain the latter hypothesis relies on midazolam's high lipid solubility at physiologic pH.<sup>10</sup> As midazolam diffuses into the slice, its affinity for the lipophilic membranes of the cells at the surface of the slice slows its passage to the cells deep within

the slice; DMSO could increase the affinity of midazolam for the aCSF in the interstitial space of the slice, thereby enhancing its penetration.

Past experiments have shown that the decrease in ATP levels during anoxia correlates with the irreversible damage found after anoxia.<sup>2,14</sup> Indeed, if an agent reduces the decrease in ATP during anoxia, there is better recovery after anoxia.<sup>2,14,22,34</sup> Midazolam pretreatment allowed significantly better maintenance of ATP levels in the CA1 region of the hippocampal slice during 5 min of anoxia. This is the same anoxic period after which the pyramidal cells of the CA 1 region recovered their electrophysiologic activity when treated with midazolam. In untreated slices, these cells did not recover from this duration of anoxia. Thus, we have found a correlation between midazolam's ability to protect electrophysiologic activity and its maintenance of ATP levels, suggesting that one mechanism of midazolam's protective effect is to maintain ATP levels.

High intracellular calcium levels have been implicated as a possible cause of anoxic damage.<sup>35,36</sup> Calcium levels have been shown to increase in cells during anoxia,<sup>37</sup> and blocking calcium influx with cobalt and magnesium can protect against anoxic damage *in vitro*.<sup>15,22</sup> Taft and DeLorenzo<sup>18</sup> and Johansen *et al.*<sup>19</sup> demonstrated that high concentrations of benzodiazepines could block calcium entry into mammalian nerve terminals and leech neurons. We found that midazolam significantly reduced the net calcium influx measured during anoxic conditions to a level lower than in untreated, normoxic CA1 regions. This may indicate that midazolam causes a direct block of calcium influx during normoxia that persists during anoxia. The effect could also be due to an improved maintenance of ATP, which would improve the pumping of calcium out of the cell. Our results do not distinguish between these two possibilities; however, they do indicate that a mechanism of midazolam's protection is a reduction of net calcium influx during anoxia.

The hippocampal slice is a useful model for studying anoxic damage since it allows for the precise control of a number of variables that confound *in vivo* studies, such as brain temperature, reoxygenation after the insult, ancillary drugs needed to maintain intact animals, and secondary effects of agents on the cerebral circulation. The slice preparation allows comparison of physiologic and metabolic values from the same small region of the brain; this is important because different brain regions show different susceptibilities to anoxia.

It is clear that finding a protective effect *in vitro* does not establish that an agent will protect *in vivo* or clinically. However, it is useful to compare our results with those from *in vivo* animal studies to establish whether the same basic mechanisms are important for both preparations. One early study that screened for protective effects<sup>38</sup> and

a later study that used more sophisticated techniques<sup>¶</sup> found that midazolam protected against anoxic or ischemic damage *in vivo*. These results are similar to those we found *in vitro*. Thus, our results do not appear to be an anomaly of *in vitro* techniques but correlate with *in vivo* studies and add insight into the mechanism by which midazolam protects against damage.

We initially chose 1  $\mu\text{M}$  midazolam because this is an effective clinical plasma concentration for exerting anesthetic effects.<sup>39,40</sup> We did not see significant protection at this concentration. A much higher concentration (100  $\mu\text{M}$ ) was needed to demonstrate protection. *In vivo*, a high concentration of midazolam, measured in one study as 53  $\mu\text{g}/\text{ml}$  (120  $\mu\text{M}$ ),<sup>41</sup> was required to maximally reduce the cerebral metabolic rate to between 55 and 75% of control levels.<sup>38,41,42</sup> This concentration is similar to the high dose of midazolam (100  $\mu\text{M}$ ) we used. The reduced metabolic rate found *in vivo* may correspond to the reduced decrease in ATP during anoxia that we found with midazolam. *In vivo* animal studies indicate that high concentrations of midazolam do not adversely affect phosphocreatine, ATP, or lactate levels in the brain and that the EEG changes induced by this concentration can be reversed.<sup>41</sup> Further animal experiments are needed to confirm that, given ventilatory support, this concentration of midazolam is not toxic.

In conclusion, we found that high concentrations of midazolam protect against anoxic damage in the *in vitro* hippocampus and that both better maintenance of ATP and reduced net influx of calcium during anoxia may contribute to this protective effect.

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¶ Baughman VL, Hoffman WE, Miletich DJ, Albrecht RF: Cerebral metabolic depression and brain protection produced by midazolam and etomidate in the rat. *J Neurosurg Anesthesiology* 1:22-28, 1989.

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