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Protection Against Sarin-Induced Seizures in Rats by Direct Brain Microinjection of Scopolamine, Midazolam or MK-801

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Abstract Control of seizure activity is critical to survival and neuroprotection following nerve agent exposure. Extensive research has shown that three classes of drugs, muscarinic antagonists, benzodiazepines, and *N*-methyl-D-aspartate antagonists, are capable of moderating these seizures. This study began to map the neural areas in rat brain that respond to these three drug classes resulting in anticonvulsant effects. Drugs of each class (scopolamine, midazolam, MK-801) were evaluated for their ability to prevent sarin-induced seizures when injected into specific brain areas (lateral ventricle, anterior piriform cortex, basolateral amygdala, area tempestas). Animals were pretreated by microinjection with saline or a dose of drug from one of the three classes 30 min prior to receiving 150 μg/kg sarin, subcutaneously, followed by 2.0 mg/kg atropine methylnitrate, intramuscularly. Animals were then returned to their cages, where electroencephalographic activity was monitored for seizures. Anticonvulsant effective doses (ED_{50}) were determined using an up–down dosing procedure over successive animals. Scopolamine provided anticonvulsant effects in each area tested, while midazolam was effective in each area except the lateral ventricle. MK-801 was only effective at preventing seizures when injected into the basolateral amygdala or area tempestas. The results show a unique neuroanatomical and pharmacological specificity for control of nerve agent-induced seizures.

Keywords Sarin · Seizures · Microinjection · Anticonvulsant · Lateral ventricle · Anterior piriform cortex · Basolateral amygdala · Area tempestas

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Introduction

Organophosphorus nerve agents, such as sarin, inhibit acetylcholinesterase (AChE) within minutes of exposure in all organ systems (Shih et al., 2005). The resulting hyperactivity of the cholinergic system can be seen physically by the manifestation of toxic signs, including seizures. Without early intervention, this seizure activity can lead to severe brain damage and mortality (Shih et al., 2003). The development of neuropathology following seizures is a time-dependent process, with the amount of damage increasing rapidly as time in seizure progresses (McDonough et al., 1995).

In a model proposed by McDonough and Shih (1997), nerve agent seizures are initiated and initially maintained by cholinergic overstimulation. The seizure activity itself progressively recruits glutamatergic excitatory amino acid

(EAA) activity to control the seizure activity independent of the initiating cholinergic overstimulation. Coinciding with this model, three classes of drugs have been shown to exert substantial anticonvulsant action against nerve agent-induced seizures: anticholinergics (in particular, muscarinic receptor blockers), benzodiazepines (drugs that enhance gamma-amino-butyric acid [GABA] activity), and *N*-methyl-D-aspartate (NMDA) EAA antagonists (McDonough and Shih, 1993; Lallement et al., 1994).

Research in the field of epilepsy has suggested several areas within the limbic forebrain, such as anterior piriform cortex, basolateral amygdala, and area tempestas, as possible foci for the beginning of seizure activity. They play critical roles in the initiation, propagation, and maintenance of limbic seizures and may also form an interconnecting network for seizure generation within limbic structures (Piredda and Gale, 1985; McDonough et al., 1987; Loscher and Ebert, 1996; Bertram et al., 1998). Direct microinjection of the nerve agents soman or VX into the basolateral amygdala or area tempestas has also been shown to elicit seizures similar to those resulting from systemic administration of these agents (McDonough et al., 1987; Myhrer et al., 2008).

Focal application of convulsant compounds, as well as of treatment drugs, has proved to be an effective method for studying the pharmacology of discrete brain areas involved in seizure generation or control (Gale, 1995; Myhrer et al., 2006). Following the lead of Myhrer et al. (2006), the present study utilized the individual microinjection of three pharmacologically distinct classes of drugs effective against nerve agent-induced seizure (the anticholinergic scopolamine, the benzodiazepine midazolam, and the NMDA antagonist MK-801) into brain areas (lateral ventricle, anterior piriform cortex, basolateral amygdala, and area tempestas) to form a better understanding of the neuropharmacological mechanisms and neural pathways involved in control of nerve agent-induced seizures.

Materials and Methods

Subjects Male Sprague–Dawley rats (Charles Rivers, Kingston, NY, USA), weighing 250–350 g before surgery, were used for this experiment. Animals were individually housed in an environmentally controlled room (temperature 21±2°C, 12-h light–dark cycle) with food and water ad libitum except during experimentation. Animals were acclimated for 1 week prior to experimentation.

Materials Atropine methylnitrate (AMN), scopolamine hydrobromide, midazolam hydrochloride, and MK-801 hydrogen maleate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Buprenorphine HCl (Buprenex Injectable, 0.3 mg/ml) was purchased from Reckitt Benckiser Pharmaceuticals (Richmond, VA, USA). AMN, scopolamine, midazolam, and MK-801 were prepared in 0.9% normal saline. Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, Aberdeen, MD, USA). Sarin (300 μg/ml) was diluted in ice-cold saline prior to injection. Sarin and AMN were prepared to be administered at 0.5 ml/kg.

Surgery Surgical procedures were performed using aseptic techniques on either a Kopf stereotaxic frame (David Kopf, Tujunga, CA, USA) or Benchmark Angle Two Dual Stereotaxic with Angle Two Software version 1.0 (MyNeuroLab, St. Louis, MO, USA). Rats were surgically prepared 1 week prior to the experiment with stainless steel cortical screw electrodes to record brain electroencephalographic (EEG) activity and implanted unilaterally or bilaterally with 22-gauge guide cannulae directed toward a designated area of the brain. Animals were given buprenorphine HCl (0.05 mg/kg, subcutaneously [sc]) before surgery and upon recovery from anesthesia and allowed 7 days rest before experimentation. The lateral ventricle was used as a non-specific site control. Due to the ability of treatments to diffuse throughout the ventricle and into multiple adjacent brain structures, a single cannula was implanted in this structure. Cannulae to all other brain areas were placed bilaterally 1 mm above the targeted structures. Targeting of brain structures was accomplished using the atlas of Paxinos & Watson (2005). The following coordinates were used relative to bregma: lateral ventricle—A-P −0.8 mm, L±1.5 mm, V −4.6 mm; anterior piriform cortex—A-P +1.2 mm, L± 4.0 mm, V −7.7 mm; basolateral amygdala—A-P −2.8, L±5.0 mm, V −8.5 mm; and area tempestas—A-P +2.5 mm, L±3.0 mm, V −7.0 mm.

Experimental Procedure

EEG recording EEG recording was done using CDE 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Cambridge, UK). Animals were free to move around the cages during recording. EEG activity was monitored for 30 min prior to any treatments to establish baseline brain activity. To inject the pretreatment test drugs, animals were removed from the cage and EEG monitoring and restrained gently in a wrapped towel.

Microinjections Dummy stylets were removed and the injection cannulae were inserted into the guide cannulae. Two 10-μl syringes were each individually connected with polyethylene tubing to the injection cannulae (28-gauge)

that projected 1 mm beyond the tip of the guide cannulae. Treatment was delivered manually at a rate of 0.2 μl/min over a period of 5 min (total 1 μl/cannula) with DMP electronic micrometers (World Precision, Sarasota, FL, USA). An injection volume of 1 μl diffuses to an area of approximately 1 mm in diameter in brain tissue (Myhrer and Andersen, 2001). Injection cannulae remained in place 1 min after injection to allow for drug diffusion. After the injection was complete, dummy stylets were replaced, and the animal was returned to its cage for continued EEG monitoring.

Dose ranging followed the up–down procedure of Dixon and Massey (1981). This procedure allows a rapid concentration of testing around the dose range of interest. Using this procedure, animals were given a starting pretreatment dose of a drug, and if that dose prevented seizures, the next animal received a lower dose; if the initial dose did not prevent seizures, the next animal received a higher dose. Dosing proceeded using this rule until at least three reversals (seizure to no seizure, no seizure to seizure) occurred or the solubility of a drug in saline limited a higher concentration (see Tables 1, 2, and 3). A 0.25 $\log_{10}$ interval was used between successive doses except between the 1.45- and 0.91-μg/μl doses for scopolamine (at 0.20 $\log_{10}$ interval). This difference in dosage step intervals did not affect any statistical testing. The starting doses were subsequently lowered in the anterior piriform cortex and area tempestas groups for scopolamine due to the high sensitivity shown initially in the basolateral amygdala to this drug.

Thirty minutes following microinjection, animals were injected with sarin (150 μg/kg, sc). The peripheral muscarinic receptor antagonist AMN was given intramuscularly (2.0 mg/kg) 1 min after sarin to relieve peripheral effects. EEG was monitored for the development of seizure activity for at least 4 h following sarin injection. Seizure onset was operationally defined as the appearance of ≥10 s of rhythmic high-amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as having been protected (seizure prevented and animal survived >1 h) or not protected (seizure and/or death occurred <1 h) based on the appearance of the EEG during the first hour after sarin exposure.

Twenty-four hours after sarin exposure, animals that survived were deeply anesthetized with sodium pentobarbital (75 mg/kg, intraperitoneally) and perfused via the aorta with saline followed by 10% neutral buffered formalin. The brain was then removed, stored in 10% neutral buffered formalin, and blocked in the coronal plane using a brain matrix. It was then embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) for verification of cannulae placement. All cannulae placements were found to be within accepted areas.

Table 1 Dose range for scopolamine pretreatments

Treatment(μg/cannula)	LV	aPC	BLA	AT
45.85	X			
25.79	O	X		
14.5	O	O	X	
8.15		X	X	
4.59			X	
2.58			X	
1.45		X	O	
0.91		O	X	
0.51			X	
0.29			X	
0.16			O	

Each X or O represents one animal

LV lateral ventricle, *aPC* anterior piriform cortex, *BLA* basolateral amygdala, *AT* area tempestas, *X* no seizure, *O* seizure on

Table 2 Dose range for midazolam pretreatments

		LV	aPC	BLA	AT
Treatment(μg/cannula)	70.8		O		
	39.8		O	X	X
	22.4	O	O	X	O X
	12.5	O	X O	X X	X O O
	7.03		O	X X	O
	3.95			O X X	
	2.22			O	O

Each X or O represents one animal

LV lateral ventricle, *aPC* anterior piriform cortex, *BLA* basolateral amygdala, *AT* area tempestas, *X* no seizure, *O* seizure on

Table 3 Dose range for MK-801 pretreatments

		LV	aPC	BLA	AT
Treatment(μg/cannula)	19.67		O	O	O
	11.06		O	O	O
	6.22		O	O	O
	3.5	O	O	X	O
	1.97	O	O	O X	O
	1.11	O	O	O X	O X X
	0.63			O	X O X
	0.35				O O

Each X or O represents one animal. All animals were used in the determination of anticonvulsant ED50 values

LV lateral ventricle, *aPC* anterior piriform cortex, *BLA* basolateral amygdala, *AT* area tempestas, *X* no seizure, *O* seizure on

Statistical Analysis A Chi square analysis was used to determine differences in 24-h survival following successful and unsuccessful pretreatments. An exponential model was used to fit the data and estimate the anticonvulsant ED_{50} values and 95% confidence intervals. Graph Pad Prism statistical software was also used to run the model fitting and an R-squared value was used to determine the goodness of fit of the model. An F test was used to test the null hypothesis that one curve fit all the data sets vs different curves for each data set. Significance was defined as $p \leq 0.05$.

Results

Four animals for each brain area were pretreated with saline through microinjection and received sc saline injections in place of sarin to examine whether cannulae placements or injection volumes alone produced behavioral modifications or seizure activity. All of these animals displayed normal EEG activity following both saline injections and showed no behavioral abnormalities. Seven animals for each brain area received saline pretreatments followed by sarin injections (sarin/saline control group; total of 28 animals). Fifteen of the 28 sarin/saline control animals displayed EEG seizure activity with an average seizure onset time of 11 min 41 s after sarin injection. Sixteen of the 28 sarin/saline control animals died (8/15 [53%] of seizure animals, 8/13 [62%] of animals that did not exhibit seizures). All sarin/saline control animals exhibited severe signs of intoxication, including ataxia, mastication, hypersecretion, fasciculations, and tremors. In sarin/saline control animals that did not exhibit EEG seizures, a marked increase in EEG amplitude and frequency was recorded.

A significant relationship was found between successful prevention of sarin-induced seizures and 24-h survival: 38 of 39 animals in which pretreatment prevented seizures survived 24 h. This was statistically different from unsuccessful pretreatments in which only 12 of 52 animals survived 24 h (x^2=49.78, df=1, p<0.0001). Significant statistical differences were also found not only between the anticonvulsant ED_{50} values for each class of pretreatment within individual brain areas, but also in the anticonvulsant ED_{50} values for each individual class of pretreatment between brain areas (see Table 4). Scopolamine was the most effective overall pretreatment, providing an anticonvulsant response in each individual brain area tested. Pretreatment with scopolamine was sevenfold, fivefold, and 18-fold more effective than midazolam in the anterior piriform cortex, basolateral amygdala, and area tempestas, respectively, and sixfold more effective than MK-801 in the basolateral amygdala. However, scopolamine was nearly twofold less potent than MK-801 in the area tempestas. Midazolam was not effective in the lateral ventricle. MK-801 was not effective in the lateral ventricle or the anterior piriform cortex.

In comparing individual pretreatments between brain areas, the basolateral amygdala had the greatest sensitivity to producing an anticonvulsant response with scopolamine and midazolam. Scopolamine microinjected into the basolateral amygdala was 50-fold more potent in blocking seizures than in the lateral ventricle and greater than twofold and threefold more potent in blocking seizures than in the area tempestas and anterior piriform cortex, respectively. Midazolam microinjected into the basolateral amygdala was fourfold and sevenfold more effective than in the anterior piriform cortex and area tempestas, respec-

Table 4 Anticonvulsant ED_{50} doses of scopolamine, midazolam and MK-801

Treatment	Brain structure			
	Lateral Ventricle	Anterior Piriform Cortex	Basolateral Amygdala	Area Tempestas
Scopolamine (μg/cannula)[a]	21.00 (6.30–[c])	1.63 (0.75–[c])	0.42 (0.28–0.90)	1.01 (0.46–[c])
Systemic (mg/kg)[b]	0.07	0.011	0.003	0.007
Midazolam (μg/cannula)	NE	11.95 (6.42–77.02)	2.60 (1.79–4.72)	0.52 (0.15–[c])
Systemic (mg/kg)		0.08	0.016	0.125
MK-801 (μg/cannula)	NE	NE	2.60 (1.79–4.72)	0.52 (0.15–[c])
Systemic (mg/kg)			0.017	0.003

NE not effective

[a] Calculated ED_{50} values (with 95% confidence intervals) for prevention of sarin-induced seizures by microinjection of scopolamine, midazolam, or MK-801. A single cannula was used in the lateral ventricle; all other brain areas received bilateral cannulae.

[b] Systemic equivalency, for the anterior piriform cortex, basolateral amygdala, and area tempestas, was calculated by dividing experimental anticonvulsant ED_{50} values by the average weight of an experimental animal (300 g) and then multiplying the sum by 2 (these animals received bilateral injections), while, in the lateral ventricle, it was calculated by dividing experimental anticonvulsant ED_{50} values by the average weight of an experimental animal (300 g).

[c] Upper limit could not be determined.

tively. MK-801 microinjected into the area tempestas was fivefold more potent than when microinjected in the basolateral amygdala.

Discussion

The results of this study show that drugs having primary mechanisms of action on different receptor systems had varying anticonvulsant action against sarin-induced seizures when injected directly into distinct brain areas. Cholinergic, $GABA_A$, and NMDA EAA activity were able to be pharmacologically manipulated to prevent sarin-induced seizure activity. Varying pharmacological specificity was seen not only between treatments but also between the neuroanatomical structures in which they were administered.

The anterior piriform cortex, basolateral amygdala, and area tempestas are all known to be involved in seizure activity (Racine et al., 1973; Piredda and Gale, 1985; Bertram et al., 1998; Ebert et al., 2000), and may serve as links along a specific seizure pathway within the brain. It is likely that the differences in pharmacological specificity observed between these brain structures are due not only to the cellular makeup of the structures and their neuroanatomical connections to other brain areas, but also to the order in which they are activated following seizure initiation. Areas involved in the initiation or immediate propagation of seizure activity may show similar pharmacological specificity to that of the basolateral amygdala or area tempestas, with anticonvulsant responses to low concentrations of anti-muscarinics or NMDA antagonists.

The anticonvulsant doses of scopolamine, midazolam, or MK-801 were substantially smaller when applied directly to the specific sensitive brain areas than the doses of these drugs that have been shown to produce an anticonvulsant effect when administered systemically. For example, the anticonvulsant dose of scopolamine in the basolateral amygdala was 0.42 μg [0.84 μg total (bilateral) dose]; this would equate to a systemic dose of 0.003 mg/kg for a 300-g rat. In contrast, the systemic dose of scopolamine required to stop nerve agent-induced seizures has been reported to be 0.06–0.13 mg/kg (McDonough and Shih, 1993; Shih et al., 2007), which is substantially greater than the basolateral amygdala microinjection dose. However, the ED_{50} dose of scopolamine administered in the lateral ventricle (21 μg) would be similar to a systemic dose of 0.07 mg/kg, which is within the dose range reported to be effective systemically. The anticonvulsant ED_{50} doses of scopolamine, midazolam, and MK-801 microinjected in the anterior piriform cortex, basolateral amygdala, and area tempestas are all equivalent to or significantly lower than reported systemic doses of these compounds that are effective in controlling nerve agent-induced seizures (Braitman and Sparenborg, 1989; Shih et al., 1991, 2007; McDonough and Shih, 1993). This reinforces the contention that specific neuroanatomical structures are critically involved in nerve agent-induced seizures and that these structures have unique pharmacological thresholds for seizure control.

In this study, MK-801 was effective at preventing sarin-induced seizures when injected into the area tempestas. This result is at variance to the findings of Myhrer et al. (2008), who reported that direct injection of MK-801 into the area tempestas did not block soman-induced seizures. There are several differences between the model Myhrer et al. (2008) used and that of this study (for example, soman vs sarin, increased latency to seizure onset signifying protection vs no seizure signifying protection); therefore, further experimentation is warranted to elucidate the role of the NMDA subtype of glutamate receptors in the area tempestas for the control of nerve agent-induced seizures.

In summary, these findings further support the notion that highly specific neuroanatomical pathways are involved in nerve agent-induced seizure activity. Of the brain areas tested, the basolateral amygdala, provided the most sensitive anticonvulsant response. Examination of structural subunits and receptor properties within this area may provide potential benefits to novel drug synthesis for protection against nerve agent-induced seizures.

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