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Neuropharmacological specificity of brain structures involved in soman-induced seizures☆

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ABSTRACT

Pharmacological control of seizure activity following nerve agent exposure is critical in reducing neuropathology and improving survival in casualties. Three classes of drugs, anticholinergics, benzodiazepines and excitatory amino acid (EAA) antagonists, have been shown to be effective at moderating nerve agent-induced seizures. However, little is known about which brain structures are involved in producing the anticonvulsant response. This study evaluated drugs from each class, injected directly into one of three specific brain structures, the perirhinal cortex, the entorhinal cortex, or the mediodorsal thalamus, for their ability to modulate seizures induced by the nerve agent soman. The drugs evaluated were the anticholinergic scopolamine, the benzodiazepine midazolam, and the EAA antagonist MK-801. For each drug treatment in each brain area, anticonvulsant ED_{50} values were calculated using an up-down dosing procedure over successive animals. There was no statistical difference in the anticonvulsant ED_{50} values for scopolamine and MK-801 in the perirhinal and entorhinal cortices. MK-801 pretreatment in the mediodorsal thalamus had a significantly lower anticonvulsant ED_{50} value than any other treatment/injection site combination. Midazolam required significantly higher doses than scopolamine and MK-801 in the perirhinal and entorhinal cortices to produce an anticonvulsant response and was ineffective in the mediodorsal thalamus. These findings support the contention that specific neuroanatomical pathways are activated during nerve agent-induced seizures and that the discrete brain structures involved have unique pharmacological thresholds for producing an anticonvulsant response. This study is also the first to show the involvement of the mediodorsal thalamus in the control of nerve agent-induced seizures.

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1. Introduction

Seizures are one of a myriad of toxic signs that can occur following exposure to organophosphorus nerve agents such as sarin, soman and VX. A result of excessive cholinergic neurotransmission within the central nervous system (CNS), seizures not only complicate the treatment of nerve agent poisoning, but can also lead to extensive brain damage if uncontrolled. Neuropathology has been shown to develop after as little as 20 min of nerve agent-induced seizure activity with the extent and severity of damage increasing rapidly as time progresses (Lallement et al., 1994; McDonough et al., 1995). Pharmacological control of nerve agent-induced seizures is critical to survival following exposure and has been strongly associated with protection against the brain damage caused by these agents (Shih et al., 2003). An understanding of the brain structures and mechanisms that initiate, propagate, and modulate these seizures would, therefore, be beneficial in identifying targets for therapeutic intervention to increase survival and reduce brain damage.

The perirhinal cortex, entorhinal cortex, and mediodorsal thalamus are three brain structures that have been consistently associated with seizure activity in epilepsy. The perirhinal cortex (PRC) is the fastest structure in the brain to produce convulsive seizures from electric kindling (McIntyre et al., 1993) and it has been suggested to serve as a relay station for the generalization of seizure activity from limbic structures to the sensorimotor cortex (Kelly and McIntyre, 1996; Fukumoto et al., 2002). Lesions of this structure have also been shown to have an anticonvulsant effect against soman-induced seizures (Myhrer et al., 2008). The entorhinal cortex (EC) has also been implicated as a structure

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involved early in the initiation or propagation of nerve agent-induced seizures and shows intense labeling for the proto-oncogene c-Fos, a marker of activated neuronal cell bodies, following soman exposure (Denoyer et al., 1992; Zimmer et al., 1997). The mediodorsal thalamus (MDT) is a structure that may not only propagate and synchronize seizure activity, but also be capable of initiating seizures (Bertram et al., 1998, 2008). Additionally, this structure exhibits significant damage following prolonged seizure activity (Bertram and Scott, 2000; Bertram et al., 2001).

In a recent study, we began to map the anticonvulsant response of several brain structures to drugs from three classes: scopolamine, an anticholinergic, midazolam, a benzodiazepine, and MK-801, an EAA antagonist (Skovira et al., 2010). The anticonvulsant effect of these drugs against nerve agent-induced seizures is thought to occur primarily through their action on the M_1 muscarinic receptor, $GABA_A$ receptor, and NMDA receptor, respectively (McDonough and Shih, 1997). Although each drug was capable of eliciting an anticonvulsant response, the effectiveness of a given drug was differentially dependent upon the brain structure into which it was injected. The present study was designed to continue to map the anticonvulsant response of brain structures known to be involved in seizure activity, PRC, EC, and MDT, to treatments acting on different receptor systems against soman-induced seizures.

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats (Charles Rivers Labs., Kingston, NY), weighing 250–300 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.

2.2. Materials

Atropine methylnitrate (AMN), scopolamine hydrobromide, midazolam hydrochloride, and MK-801 hydrogen maleate were purchased from Sigma–Aldrich (St. Louis, MO). HI-6 (Lot#BN44621) was obtained from Walter Reed Army Institute of Research (Silver Spring, MD). Buprenorphine HCl (Buprenex Injectable, 0.3 mg/ml) was purchased from Reckitt Benckiser Pharmaceuticals Inc. (Richmond, VA). Isoflurane (Attane®) was purchased from Minrad Inc., Bethlehem, PA. Soman (pinacolyl methylphosphonofluoridate) was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Soman was diluted in ice-cold saline to a concentration of 360 μg/ml. AMN, HI-6, scopolamine, midazolam, and MK-801 were prepared in 0.9% normal saline. Soman, AMN, and HI-6 were administered subcutaneously (SC), intramuscularly (IM), and intraperitoneally (IP), respectively, with a 0.5 ml/kg injection volume. Scopolamine, MK-801, and midazolam were delivered via microinjection.

2.3. Surgery

Rats were anesthetized with isoflurane (3% induction, 1.5–2% maintenance; with oxygen) and placed in a stereotaxic frame. To record brain electroencephalographic (EEG) activity two stainless steel cortical screw electrodes were placed equidistant between bregma and lambda 2–3 mm from the midline in each hemisphere and a third was placed over the cerebellum. For drug administration two 22 gauge guide cannula (Plastics One, Inc., Roanoke, VA) were implanted bilaterally toward a designated area of the brain (PRC, EC, or MDT).

Targeting of brain structures was done using the atlas of Paxinos and Watson (2005). The following coordinates were used relative to bregma: PRC–A-P −3.5, L ±6.2, V −7.3; EC–A-P −6.0, L +6.9, V −8.0; and MDT – (15° off vertical) A-P −3.25, L ±2.5, V −6.13. Guide cannula were lowered 1 mm dorsal to the targeted brain site. To prevent plugging of the guide cannula dummy stylets of matching length were inserted. Cortical screw electrodes were covered with dental acrylic cement to electrically isolate the electrodes from the overlying tissue and to mechanically fix the electrodes, plug, and cannula as a unit. Buprenorphine HCl (0.05 mg/kg) was administered SC upon recovery from anesthesia. Animals were given 7 days rest before experimentation.

2.4. Experimental procedure

2.4.1. EEG recording

To monitor EEG, animals were connected to recording leads and placed in individual 25 cm × 25 cm × 45 cm plastic recording cages. EEG recording was done using CDE Model 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). EEG activity was monitored for 30 min to establish baseline brain activity prior to any treatments. After baseline recording, animals were pretreated with a test drug through bilateral microinjection into the guide cannulae. In order to inject the pretreatment test drugs, animals were removed from the cage and EEG monitoring, and restrained gently in a wrapped towel.

2.4.2. Microinjections

The dummy stylets were removed, and 28 gauge injection cannula were inserted into the bilateral guide cannula. The injection cannula projected 1 mm beyond the tip of the guide cannula. Treatment was delivered manually at a rate of 0.2 μl/min for 5 min (total injection volume was 1 μl/cannula) using DMP electronic micrometers (World Precision Instruments Inc., Sarasota, FL). Injection cannula remained in place 1 min after completion of the injections to allow for drug diffusion. The dummy stylets were then replaced, and the animal was pretreated with the oxime HI-6 (125 mg/kg, IP) to antagonize the peripheral effects of the nerve agent. Animals were then returned to a recording cage for continued EEG monitoring.

Dosing followed the up-down procedure of Dixon and Massey (1983). Using this procedure, animals were given a starting pretreatment dose of a drug, and if that dose prevented or significantly increased the latency to onset of seizures, the next animal received a lower dose; if the initial dose did not prevent or significantly increase the latency to onset of seizures, the next animal received a higher dose. Dosing proceeded using this rule until five reversals occurred (i.e., seizure to no seizure, no seizure to seizure). Pretreatment doses ranged between 0.15–81.54 μg/μl per cannula with 1.45 μg/μl as the starting dose for scopolamine and MK-801 and 14.50 μg/μl for midazolam treatment based on our previous data (Skovira et al., 2010). A 0.25 $\log_{10}$ interval was used between successive doses.

2.4.3. Nerve agent exposure

Thirty minutes following drug pretreatment, animals were injected with the nerve agent soman (180 μg/kg, SC). The peripheral muscarinic receptor antagonist AMN (2.0 mg/kg, IM) was given 1 min after soman. Animals were monitored for seizure activity for at least 4 h following exposure and again at 24 h. Seizure onset was operationally defined as the appearance of ≥10 s of rhythmic high amplitude spikes or sharp wave activity in the EEG tracing. For each animal, treatment was categorized as successful (prevention or significant increase in the latency to

onset of seizure) or not successful (no significant increase in the latency to onset of seizure) based on the overall EEG record.

A saline-treated/GD-exposed control group was not used in this experiment to reduce animal use for two reasons: (1) previous work from our laboratory using this experimental model in rats (HI-6 pretreatment [125 mg/kg, IP] followed thirty min later by soman [180 μg/kg, SC] and then immediate treatment with AMN [2.0 mg/kg, IM]) has shown 100% seizure occurrence in both cannulated and uncannulated experimental animals and (2) the seizure onset times between studies utilizing GD are very similar (i.e. between 4 and 7 min) (Shih, 1990; Shih et al., 1991; Capacio and Shih, 1991; McDonough and Shih, 1993; McDonough et al., 1998; Myhrer et al., 2006, 2007, 2008, 2010). For this study, a significant increase in the latency to seizure onset was defined as occurrence of seizure greater than 25 min after soman challenge. This represents a time greater than 3 standard deviations (SD) beyond the average seizure onset time for this model and outside the range of the maximum seizure onset time seen in cannulated untreated animals using this model (Ave = 6 min 51 s; SD = 3 min 49 s; $N = 100$; Range = 2 min 15 s −19 min 7 s; Guarisco et al., 2009). Therefore, a lack of seizure activity or delay in seizure onset until after this time could be confidently attributed to the effects of the pretreatment drug.

2.4.4. Histology

Twenty-four hours after soman exposure, animals that survived were deeply anesthetized with sodium pentobarbital (75 mg/kg, IP) and perfused via the aorta with saline followed by 4% paraformaldehyde. The brain was then removed and stored for 4 h in 4% paraformaldehyde, then transferred to a 30% sucrose solution, and frozen in isopentane chilled to −70 °C. The brain was then sectioned in the coronal plane, mounted on glass slides, and stained with hematoxylin and eosin (H & E) for verification of cannula placement. Cannulae placements for all animals included in this study were reconstructed to be within acceptable areas.

2.5. Data analysis

Anticonvulsant ED_{50} values and 95% confidence intervals (C.I.) were determined using a Microsoft Excel (2007) macro developed in-house at USAMRICD by Mr. Rich Sweeney, which utilizes the calculations as set forth by Dixon and Massey (1983) for the up-down procedure. Anticonvulsant ED_{50} values were determined to be significantly different if their 95% C.I. did not overlap.

3. Results

3.1. Signs of intoxication

All animals showed severe signs of nerve agent intoxication following soman injections. Within minutes of exposure and prior to seizure onset, animals exhibited a "staring" behavior usually in association with rapid rhythmic mouth and facial clonus. These toxic signs most commonly progressed into full body tremors, Straub tail, and rhythmic head nodding. Excessive salivation and splaying of the hind legs or loss of postural control was typically observed in conjunction with high frequency seizure activity. Lacrimation, forelimb clonus, rearing, and explosive running and bouncing were also observed, but were less frequent.

Table 1
Anticonvulsant responses/total N (treatment dose range in μg/cannula) for pharmacologically distinct anti-seizure drugs in rat brain.

Treatment	Brain structure		
	Mediodorsal thalamus	Perirhinal cortex	Entorhinal cortex
Scopolamine	3/8 (1.45–8.15)	4/11 (1.45–14.50)	4/10 (1.45–14.50)
Midazolam	2/8 (14.50–81.54)	3/7 (8.15–25.79)	4/9 (14.50–81.54)
MK-801	7/12 (0.15–1.45)	4/8 (1.45–8.15)	4/10 (1.45–8.15)

Table 2
Anticonvulsant ED_{50} values (95% limits) for pharmacologically distinct anti-seizure drugs in rat brain.

Treatment (μg/cannula)	Brain structure		
	Mediodorsal thalamus	Perirhinal cortex	Entorhinal cortex
Scopolamine	6.38 (3.33–12.24)	5.84 (3.53–9.68)	8.22 (4.50–15.02)
Midazolam	ND	19.41 (10.62–35.49)	39.70 (22.59–69.81)
MK-801	0.36 (0.21–0.61)	4.41 (2.41–8.06)	5.47 (3.11–9.61)

3.2. Effects of pretreatments

Each drug treatment was capable of producing an anticonvulsant response when microinjected within the brain structures tested (Table 1 and Fig. 1). Anticonvulsant ED_{50} values were determined following five reversals using the up-down procedure and are shown in Table 2. There was no difference in the anticonvulsant ED_{50}s of scopolamine among the three brain regions. There was no difference in the anticonvulsant ED_{50}'s of midazolam treatment between the PRC and EC, while an anticonvulsant ED_{50} for midazolam treatment in the MDT could not be determined. MK-801 treatment in the MDT provided an anticonvulsant response at a significantly lower ED_{50} than in the PRC or EC. This resulted in an anticonvulsant ED_{50} 12- and 15-fold less than that determined for the PRC and EC, respectively. There was no difference in the anticonvulsant ED_{50}'s of MK-801 between the PRC and EC.

The three brain structures showed differential sensitivities to the anticonvulsant effects of the three test drugs. Microinjections into the MDT had significantly different anticonvulsant effectiveness among treatments. MK-801 produced an anticonvulsant ED_{50} that was 17-fold lower than that of scopolamine treatment, while an ED_{50} for midazolam could not be determined. Within the PRC and EC there was no difference in the anticonvulsant effectiveness

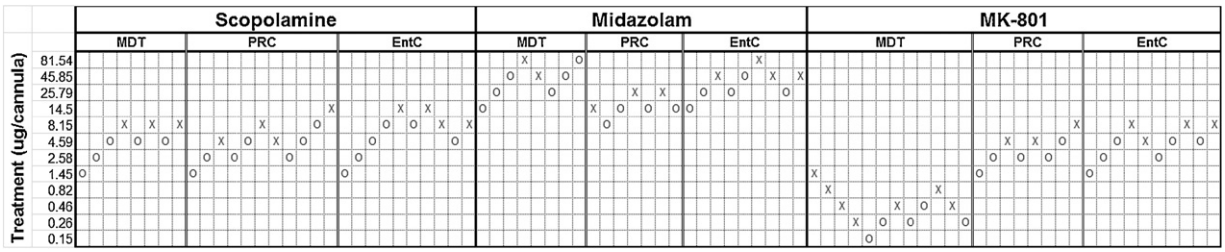

Fig. 1. Effects of scopolamine, midazolam, or MK-801 injected into the mediodorsal thalamus (MDT), perirhinal cortex (PRC), or entorhinal cortex (EntC), from left to right columns, following the up-down procedure of Dixon and Massey (1983). Rats were pretreated with a drug via guide cannula into specific brain areas 30 min prior to soman (180 μg/kg, SC) challenge. O = unsuccessful treatment (seizure not prevented); X = successful treatment (latency to seizure onset significantly increased).

of scopolamine or MK-801. Both scopolamine and MK-801 treatment, however, had significantly lower anticonvulsant ED_{50} values than that of midazolam treatment within these two areas.

4. Discussion

Evidence from epilepsy research suggests that seizures occur through specific neuroanatomical pathways or networks (Gale, 1992; Löscher and Ebert, 1996). The various discrete brain structures that comprise these networks may also serve different functions in regards to initiating, propagating, or sustaining seizure activity (White and Price, 1993a,b; McIntyre and Gilby, 2008). Research has shown that similar pathways and structures are likely involved in nerve agent-induced seizure activity (McDonough et al., 1987; Skovira et al., 2010; Myhrer, 2010).

The results of this study provide further evidence that highly specific neuroanatomical pathways are involved in nerve agent-induced seizures. Pharmacological intervention at any point in these pathways seems to provide anticonvulsant benefits with specificity as to which brain structures respond to various treatments. It is also evident that a certain threshold for each treatment must be reached within these structures to produce an anticonvulsant response. Although all structures that respond to a certain treatment may contribute in some capacity to the anticonvulsant effect following systemic administration, structures with the lowest thresholds for response as identified through microinjections are likely the primary sites of action for these treatments in the brain. Here we will refer to these brain structures as "primary anticonvulsant response centers" (i.e., structures most sensitive to producing an anticonvulsant response from a low dose of a specific drug treatment). In this study, we have also identified three brain structures, PRC, EC, and MDT, in addition to those previously identified (basolateral amygdala [BLA], area tempestas [AT]), that respond to glutamatergic antagonism to produce an anticonvulsant effect against nerve agent-induced seizures (Myhrer and Aas, 2008; Skovira et al., 2010). Further, this is the first time that the MDT has been implicated for its ability to modulate these seizures. Below we discuss the implications of these findings in providing a directed path for future research on controlling nerve agent-induced seizures.

Organophosphorus nerve agents inhibit the enzyme AChE, which results in excessive ACh accumulation at neuronal junctions and facilitates seizure initiation due to cholinergic overstimulation. It is therefore no surprise that the potent anticholinergic scopolamine, delivered in microinjections, has proven effective as an anticonvulsant against nerve agent-induced seizures in every brain structure that we have tested to this point. The anticonvulsant ED_{50} values determined in this study for scopolamine in the PRC, EC, and MDT are markedly higher doses than those we previously determined for the BLA, anterior piriform cortex (aPC), or the AT (0.42, 1.63, 1.01 μg/μl, respectively) (Skovira et al., 2010). We can therefore begin to identify possible primary anticonvulsant response centers for scopolamine, such as the AT, aPC, and BLA. These structures possibly represent the main sites of action for this drug within the brain when scopolamine is administered systemically. Structures requiring higher doses, such as the PRC, EC, and MDT, may serve in a secondary capacity, providing additional anticonvulsant effects when the proper threshold of the drug is reached within that structure.

The difference in doses needed to produce an anticonvulsant response appears to be the function of an inherent threshold unique to each individual structure and is independent of the nerve agent used to elicit the seizure. For example 1 μg/μl of scopolamine microinjected into the AT will produce an anticonvulsant response from this structure regardless of whether the seizures are elicited by sarin or soman (Myhrer et al., 2008; Skovira et al., 2010). This observation shifts the focus for treatment from a more general outlook onto the inherent properties of individual brain structures and their unique threshold to produce a response. Since pharmacological intervention within a structure at any point within these pathways appears to provide anticonvulsant effects, targeting brain structures that respond to the lowest threshold of a given drug to produce this response provides a novel direction for the development of new treatments in regards to reducing global side effects while maintaining anticonvulsant effectiveness.

Further support for the notion that each structure has a unique threshold for producing an anticonvulsant response is found by examining the results of scopolamine microinjections in the PRC. Myhrer et al. (2010) reported that 1 μg/μl of scopolamine microinjected into the PRC provided no anticonvulsant effect against soman-induced seizures. In our current study, the lowest dose of scopolamine in the PRC that provided an anticonvulsant effect was 4.59 μg/μl. This may represent the lowest dose needed to reach the threshold for producing an anticonvulsant response with scopolamine in the PRC.

As might be expected, the threshold for response in any particular brain structure is also dependent upon the neurotransmitter system that is being targeted by the treatment. $GABA_A$ modulators such as midazolam appear to require markedly higher doses than scopolamine to produce an anticonvulsant response from a given brain structure. The anticonvulsant ED_{50}'s determined in our current study for midazolam in the PRC and EC are similar to those determined for the aPC and AT (11.95 and 18.73 μg/μl, respectively) in our previous study (Skovira et al., 2010). The anticonvulsant ED_{50} value for midazolam in the BLA (2.43 μg/μl) is 4- to 16-fold lower than the values of all other structures we have tested. This suggests that the BLA may also be a primary anticonvulsant response center to midazolam treatment.

The possible involvement of the glutamatergic system in the initiation or propagation of nerve agent seizures is much less delineated than that of the cholinergic system. The early phase ($\leq$5 min after onset) of seizure activity following nerve agent intoxication is believed to be controlled primarily by cholinergic events (McDonough and Shih, 1997). The extent to which glutamatergic signaling contributes to this early phase of seizure activity is unknown. The glutamatergic antagonist MK-801 has been shown to be an effective anticonvulsant against soman-induced seizures prior to and during this initial phase of seizure activity (Braitman and Sparenborg, 1989; Shih, 1990; McDonough and Shih, 1993). This suggests almost immediate involvement of the glutamatergic system in soman-induced seizure activity. Additionally, several studies utilizing intracranial microdialysis techniques have also shown a rise in extracellular glutamate during the early phase of seizure activity following soman administration (Wade et al., 1987; Lallement et al., 1991). It still remains to be determined if the involvement of the glutamatergic system is restricted primarily to controlling the seizure activity. Although there is a possibility that the glutamatergic system in some capacity may contribute to the initiation of seizure activity, it more likely plays a role in the propagation of the seizures.

Differential responses to microinjections of EAA antagonists have been observed against nerve agent-induced seizures. Myhrer et al. (2010) found that the glutamatergic antagonist ketamine, which acts on the NMDA receptor, was ineffective against soman-induced seizures when infused into the PRC. Myhrer concluded that blocking of the NMDA receptor by ketamine in the PRC must be supported by muscarinic antagonism to produce an anticonvulsant effect. This conclusion was derived from the efficacy of procyclidine (a drug with both antimuscarinic and antiglutamatergic properties) against the same challenge, but he stipulated that procyclidine may be a more potent NMDA antagonist than ketamine. In addition, when administered systemically, ketamine

also appears to require the support of an antimuscarinic to obtain anticonvulsant efficacy (Shih et al., 1999; Dorandeu et al., 2005).

MK-801 is a potent NMDA antagonist which has been shown to be effective against soman-induced seizures when administered systemically with antimuscarinic support (Braitman and Sparenborg, 1989; Shih, 1990; McDonough and Shih, 1993). However, MK-801 does not appear to require similar antimuscarinic support to produce an anticonvulsant effect when administered centrally. Co-administration of antimuscarinics with MK-801 more likely suppresses unwanted peripheral interactions of the drug when it is given systemically (Shih et al., 1991). Microinjections of MK-801 directly into the brain eliminate the unwanted peripheral side effects from systemic administration of this drug and clearly demonstrate the ability of NMDA signaling alone to modulate the initial phase of seizure activity. In our current study, the PRC and EC had anticonvulsant ED_{50}'s for MK-801 similar to that previously determined for the BLA (2.60 μg/μl) against sarin-induced seizures (Skovira et al., 2010). The anticonvulsant ED_{50} values for the aforementioned structures are 5- to 15-fold greater than that determined for MK-801 in the MDT (0.36 μg/μl) or previously determined for the AT (0.52 μg/μl). Therefore, the AT and MDT may serve as primary anticonvulsant response centers for MK-801 in the rat brain.

While the perirhinal and entorhinal cortices have previously been implicated (Myhrer, 2007), our study is the first to show the involvement of the MDT in the control of nerve agent seizures. The MDT has well known and widespread reciprocal connections to limbic structures and the basal ganglia (Groenewegen, 1988). Many of the structures with which it shares these reciprocal connections are also known to be involved in seizure activity. It is because of these extensive connections that the MDT has been suggested as a central synchronizer of limbic seizures (Bertram et al., 2001). The MDT has also been shown to be involved from the onset of, and capable of initiating, kindled limbic seizures as well as generalizing seizure activity more rapidly than from the amygdala or hippocampus (Bertram et al., 2001, 2008). Because multifocal onset of seizure activity is a possibility in epilepsy and following nerve agent exposure especially, the role of the MDT and its connectivity make it a prime target for anticonvulsant intervention. Decreasing excitatory transmission within the MDT using the NMDA antagonist 2-amino-7 phosponoheptanoate has been shown to provide potent anticonvulsant effects against systemic pilocarpine convulsions (a model of cholinergic overstimulation) (Patel et al., 1988). In this study we showed that pharmacological manipulation of not only the NMDA receptor but also the muscarinic receptor signaling within the MDT is capable of modulating nerve agent-induced seizures.

4.1. Conclusions

Our findings support the contention that specific neuroanatomical pathways are activated during nerve agent-induced seizures and that the discrete brain structures involved have unique pharmacological thresholds for producing an anticonvulsant response. Knowledge of which brain structures serve as primary anticonvulsant response centers for anticonvulsant drugs has value in new drug testing and development. Future research identifying receptor subtypes, receptor subunit composition, or unique cellular markers may provide the possibility of specifically targeting such structures while causing fewer global side effects and maintaining anticonvulsant effectiveness against nerve agent-induced seizures.

Conflict of interest

None.

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