

## Anticonvulsant Efficacy Of Pharmacological Agents Microinfused Into Medial Septum Of Rats Exposed To Soman

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### ABSTRACT

The medial septum is considered to belong to a group of brain structures termed as seizure controlling sites. The septal area is dominated by both cholinergic input and output. The activity of the cholinergic neurons in the medial septum can, however, be modified by glutamatergic antagonists and  $\gamma$ -aminobutyric acid (GABA)ergic agonists in addition to cholinergic antagonists. The purpose of this study was to examine whether a single dose of anticholinergics (atropine, scopolamine, procyclidine), antiglutamatergic drug (ketamine), or GABA enhancing drug (muscimol) microinfused (1  $\mu$ L) into the medial septum may produce anticonvulsant effects in rats exposed to soman (1.3 x LD<sub>50</sub> subcutaneously). The results showed that infusion of atropine, scopolamine, or procyclidine into the medial septum increased latency to seizure onset, but did not prevent seizures, whereas ketamine and muscimol had no anticonvulsant impact. Repeated microinfusions of atropine into the medial septum after seizure onset

temporarily reduced convulsive activity, but did not terminate it. Microinfusion of soman into the medial septum resulted in proconvulsive reactions only. Compared with other seizure controlling sites (area tempestas, substantia nigra) the medial septum seems to have more moderate anticonvulsant potency against soman.

## **INTRODUCTION**

The nerve agent soman is a very toxic organophosphate that irreversibly inhibits acetylcholinesterase, the enzyme that hydrolyses acetylcholine. High levels of cholinergic activity result in respiratory dysfunction, prolonged seizures, and death. The progression of events after nerve agent poisoning triggering seizures/convulsions can be divided into three phases. An early phase lasting from the time of exposure to about 5 min after seizure onset is dominated by excessive cholinergic activity, followed by a transitional phase of cholinergic and glutamatergic hyperactivity, and, finally a predominantly glutamatergic phase after about 40 min [McDonough and Shih, 1997; Lallement et al., 1992a]. The times of these phases are primarily based on experiments in rats and guinea pigs.

Medical treatment immediately after exposure to nerve agent is important to avoid death or brain damage among survivors. Strategies have focused on pretreatment with carbamate cholinesterase inhibitors, such as pyridostigmine, to shield a portion of the acetylcholinesterase from irreversible inhibition by the nerve agent. Subsequent therapeutic treatment with an anticholinergic drug, like atropine sulfate, has been used along with an oxime to reactivate any unaged inhibited enzyme. Even if such treatment regimen can increase the survival rate significantly, it does not effectively reduce nerve agent-induced seizure activity resulting in brain lesions. Therefore, efforts in searching for countermeasures have targeted drugs assuring cholinergic and glutamatergic antagonism along with  $\gamma$ -aminobutyric acid (GABA)ergic agonism [McDonough and Shih, 1997]. This search strategy, however, does not seem to be based on a rational screening foundation, because any drug with the above properties appears relevant for testing. In order to shorten the list of candidate drugs, identification of seizure controlling sites should be made, as well as specification of critical receptor types in the same structures, as has been done in epilepsy research.

Cholinergic innervation is seen throughout the entire brain by way of three major projection systems. One system sends axons from the nucleus basalis magnocellularis to the whole cortical mantle [Wenk et al., 1980]. Another system complex consisting of the medial septal nucleus, diagonal band nucleus and preoptic magnocellular area projects to the hippocampal region, amygdala, and piriform, insular, cingular, and entorhinal cortices [Woolf et al., 1984]. A third system arising in the dorso-lateral tegmental nucleus of the brain stem innervates the medial septal nucleus and the vertical and horizontal limbs of the diagonal band area [Woolf and Butcher, 1986]. Thus, the septal area appears to make up a nodal point for cholinergic input and output systems. The innervated regions are provided with both muscarinic and nicotinic receptors, which are differently distributed in the rat brain [London et al., 1985; Spencer et al., 1986].

The medial septum-diagonal band complex innervates the hippocampal region and a number of cortical structures. Both distribution and morphology of labeled efferents resemble those found for cholinergic innervation [Gaykema et al., 1990]. This connectivity permits septal neurons to affect both hippocampal and cortical excitability by switching between excitability states [Bland et al., 1999]. Spaced, repeated low-intensity electrical stimulation (kindling) of the medial septum evokes seizure activity [Baxter et al., 1991]. Infusion of naloxone into the medial septum results in convulsive seizures by increasing septo-hippocampal cholinergic activity [Mizuno and Kimura, 1996]. The cholinergic activity in the latter pathway is decreased by infusion of the glutamatergic antagonist, D-2-amino-5-phosphonopentanoic acid (AP5), into the medial septum [Puma et al., 1996]. Septo-hippocampal cholinergic activity is also reduced by infusion of the GABAergic agonist, muscimol, into the medial septum [Yamamoto et al., 2007]. Inasmuch as soman-induced seizures are associated with excessive cholinergic activity, microinfusion of cholinergic antagonist, glutamatergic antagonist, or GABAergic agonist into the medial septum may assure anticonvulsant efficacy.

In a previous study, we have shown that aspiration lesion of the medial septum produces anticonvulsant effects against soman-induced seizures in rats [Myhrer et al., 2007]. The purpose of the present study was to examine the potential anticonvulsant impact of intraseptal infusion of either the cholinergic antagonist, atropine or scopolamine, the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor antagonist, ketamine, the anti-Parkinson agent, procyclidine, that

exerts both cholinergic and glutamatergic NMDA receptor antagonism, or the GABA<sub>A</sub> agonist, muscimol, in rats exposed to soman. The drugs were infused into the medial septum 20 min before a subcutaneous soman dose of 1.3 x LD<sub>50</sub>, which ensures seizures in all rats. Prevention of seizures/convulsions or increased latency to onset of seizures was used as the measure of anticonvulsant effects. Additionally, soman was microinfused into the medial septum to investigate whether this area may serve as a trigger site for nerve agent-induced seizures.

## **MATERIALS AND METHODS**

### **Animals**

Male Wistar rats from a commercial supplier (Møllegaard Breeding Laboratories, Denmark) weighing 300-330 g (about 90 days old) at the time of surgery were used as subjects. The experiments were approved by the National Animal Research Authority. Six groups of rats, with eight animals in each, received bilateral microinfusions (anticonvulsant drugs or vehicle) into the medial septum. Six additional rats received microinfusion of soman into the medial septum. The animals were housed individually and had free access to commercial rat pellets and water. The rats were handled individually four days preoperatively and four days postoperatively, being allowed to explore a table top (80 x 60 cm) for three minutes per day. The climatized vivarium (21 °C) was illuminated from 0700 to 1900 h.

### **Surgery**

The rats were anesthetized intraperitoneally with diazepam (10 mg/kg) and fentanyl fluanisone (2 mg/kg). Lidocain liniment was applied to the periost. The rats were implanted stereotaxically (flat skull) with the guide cannula aimed at the medial septum. The guide cannula (25 gauge) was 0.5 mm diameter and 11 mm long. The upper part of the cannula was roughened in order to improve the grip of the dental cement (Durelon; ESPE, Seefeldt, Germany), which was anchored to the skull by steel screws. The point of insertion was 0.5 mm anterior to bregma and 0.5 mm lateral to the midline. The cannula was lowered with an angle of 5° (aimed at the medial plane) and the depth was 5 mm from top of the skull. A cannula 0.3 mm in diameter and 12 mm long (30 gauge) was fitted into the guide cannula and

protruded 1 mm beyond the latter one. The infusions were made by means of a microinjection pump (Model CMA 100, Carnegie Medicine AB, Stockholm, Sweden). To prevent plugging of the indwelling cannulas, smaller cannulas (30 gauge) with a cut and bent top were inserted to a depth of 10 mm. The rats were allowed to recover eight days before experimentation.

### **Histology**

The injection sites were marked with 1  $\mu$ L of 4 % methylene blue in saline 1 mm beyond the tip of the implanted cannulas. This marking was carried out immediately after death due to soman poisoning or before decapitation. The brains were removed and stored in 10 % formalin and dehydrated before being embedded in paraffin. The sections were made frontally 5  $\mu$ m thick and stained with hematoxylin and eosin.

### **Drug administration**

Atropine sulfate (100  $\mu$ g), scopolamine bromide (1  $\mu$ g), procyclidine hydrochloride (6  $\mu$ g), and muscimol hydrobromide (120 ng), all purchased from Sigma (St Louis, MO, USA), as well as ketamine hydrochloride (50  $\mu$ g) (Ketalar<sup>®</sup>, 50 mg/mL) were all given as in previous microinfusion studies [Myhrer et al., 2006b; 2008a]. Soman (1  $\mu$ L) was administered intracranially via infusion into the medial septum in a concentration of 11 nmol in 1  $\mu$ L [McDonough et al., 1987]. All drugs as well as saline were given in 1  $\mu$ L over 1 min while the rats were gently held, and the cannula remained in position for an additional 0.5 min before retraction. Twenty minutes following microinfusions [Denoyer et al., 1992; Lallement et al., 1992b] the rats received 1.3 x LD<sub>50</sub> of soman (100  $\mu$ g/kg) subcutaneously.

The injection volume of 1  $\mu$ L was used to ensure optimal anticonvulsant impact of the drugs. Infusion of 1  $\mu$ L of 4% methylene blue in saline (0.9%) into the temporal or entorhinal cortices invades an area of about 1x1 mm [Myhrer and Andersen, 2001], as an indication of invasion extent in the brain tissue. The dorso-ventral extent of the medial septum is more than 1 mm, thus the injection volume is small enough to stay within the medial septum.

## **EEG**

The screws used for anchoring the indwelling cannulas served as electrodes. The screw in the left hemisphere was lowered 1 mm into the parietal cortex. The contralateral screw (in the skull) served as ground. The rats were connected with the recording polygraph (Grass Model 7P5A) with alligator clips and leads. Baseline EEG activity was monitored 15 min before injection of drugs. EEG recordings were made during seizures/convulsions or incapacitation in response to soman, and 24 h following exposure to soman. Seizure activity was defined as continuous high amplitude rhythmic spike or sharp wave activity. The rats were situated in their home cages (50 x 30 x 15 cm) during the recordings.

## **Observation of animals**

Each rat was observed for overt behavioral changes and signs of intoxication. In the rats that convulsed, hypersecretion was seen from moisture of the lips and nose. Unconsciousness was determined by loss of both righting and corneal reflexes. The rats that received the microinfusion of soman were observed for convulsions/seizures and visible signs of intoxication continuously for the first 1—2 h and then 10 min at 24 h after soman exposure. When the anticonvulsant effects of drugs did not prevent seizures, the rats were euthanized unless they died spontaneously.

## **Statistics**

The measured time from the injection of soman to either the onset of seizures/convulsions or the lack of seizures measures the effectiveness of the drugs to prevent and terminate seizures. None of the drugs and doses were able to prevent seizures, i.e., all the rats seized, therefore parametric statistics were applied. Overall analyses were carried out with parametric one-way analysis of variance (ANOVA). Group comparisons were made with Newman-Keuls multiple comparison tests. Computations were made with the Prism statistical software program (GraphPad Software CA, USA).

## **RESULTS**

### **Histology**

The tracks from the guide cannulas were well marked. Acceptable sites of infusions were reconstructed to be within the medial septum for all rats included in this study.

### **Effects of drugs**

Microinfusion of cholinergic antagonists into the medial septum resulted in evident anticonvulsant effects (table 1). The anticonvulsant effects of microinfusions into the medial septum of rats intoxicated by soman are measured in terms of latency to onset of seizures and are shown in table 1. One-way ANOVA revealed a reliable treatment effect among the groups ( $F(5,42) = 9.098$ ,  $P < 0.0001$ ). The Newman-Keuls multiple comparison tests showed that the atropine and scopolamine groups had significantly longer latencies than the saline group ( $P < 0.001$ ), i.e., the time to onset of seizures was longer. Also the procyclidine group displayed longer latencies than the saline group ( $P < 0.05$ ). Furthermore, the atropine and scopolamine groups had longer latencies than both the ketamine and muscimol groups ( $P < 0.01$ ). The procyclidine-treated rats also had longer latencies than the ketamine- and muscimol-treated animals ( $P < 0.05$ ). Ketamine and muscimol did not prevent seizures.

The rats infused with atropine in the medial septum received additional injections of atropine (100  $\mu\text{g}$ ) every 20 min following seizure onset. This post-treatment reduced epileptiform activity and attenuated motor activity, but the convulsive seizure activity recurred 10 – 15 min after each injection, and all animals died within one hour.

### **Effects of soman**

Microinfusion of soman into the medial septum ( $N = 6$ ) resulted in increased locomotor activity after 1 – 4 min. Frenetic jumping bouts along with occasional circling and/or wet dog shakes occurred 17 – 36 min following soman injection. No other signs of proconvulsive reactions were observed. Locomotor activity declined after 40 – 50 min. Twenty-four hours after exposure to soman the rats apparently behaved normally.

## **DISCUSSION**

The results from the present study showed that microinfusion of atropine, scopolamine, or procyclidine (cholinergic and glutamatergic NMDA antagonism) into the medial septum were effective against soman-induced seizures, whereas ketamine and muscimol were not. Repeated infusions of atropine into the medial septum after seizure onset markedly attenuated convulsive activity; but they were not sufficient to end seizures. Injection of soman into the medial septum did not result in tonic-clonic convulsions, but proconvulsive behavior, such as jumping bouts accompanied by circling or wet dog shakes, was seen under these conditions.

Blocking of cholinergic receptors in the medial septum appears to assure anticonvulsant efficacy. The lack of anticonvulsant effect of ketamine suggests that anticholinergic impact of procyclidine was the active property of the latter drug. Neither glutamatergic nor GABAergic agents seem to have an influence upon soman-induced seizures. In previous studies of intraseptal application of atropine, both pretreatment with a single dose and post-treatment with plural injections eventually terminated soman-generated seizures [Denoyer et al., 1992; Lallement et al., 1992b]. However, in the latter studies, the subcutaneous dose of soman was relatively low ( $0.9 \times LD_{50}$ ). The difference in soman dose may explain why a similar result was not achieved with the present rats that were additionally treated with atropine infusions after exposure to a soman dose of  $1.3 \times LD_{50}$ .

The present study showed that the medial septum is not a particularly sensitive trigger site for seizures, because infusion of soman evoked proconvulsive responses only. This result is in agreement with a previous finding that injections of VX into the medial septum cause hyperactivity only [McDonough et al., 1987]. On the other hand, soman microinfused into area tempestas, perirhinal cortex, or posterior piriform cortex can generate full tonic-clonic convulsions [Myhrer et al., 2008a; Myhrer and Enger, unpublished data].

Among seizure controlling sites, the anticonvulsant potency of area tempestas appears more powerful than that of the medial septum. Both atropine and scopolamine microinfused into area tempestas prevented seizures in 60—70 percent of the rats exposed to a soman dose of  $1.3 \times LD_{50}$  [Myhrer et al., 2008a], whereas none were fully protected in the present study. These differences may be associated with the strategic localization of area tempestas. The latter structure has direct connections with the perirhinal and piriform cortices, thus making up a

critical link in the propagation of limbic seizures evoked in area tempestas [Halonen et al., 1994].

The medial septum projects massively to the hippocampal region and entorhinal cortex [Gaykema et al., 1990]. However, hippocampal or entorhinal aspiration lesions do not result in anticonvulsant efficacy against soman-induced convulsions, whereas lesions in the perirhinal cortex or posterior piriform cortex assure anticonvulsant impact [Myhrer et al., 2008b]. Hence, the present positive findings of intraseptal infusions of anticholinergics may be related to an alternative explanation of septal connections, because the horizontal limb of the diagonal band nuclei sends massive projections to the posterior piriform cortex and moderate projections to the perirhinal cortex [Gaykema et al., 1990]. Septal microinfusion of anticholinergic agents might have invaded the diagonal band nuclei, or the cholinergic antagonism in the medial septum may have influenced the neuronal activity in the diagonal band nuclei, since the latter structure receives numerous afferents from the medial septum [Gaykema et al., 1990; Swanson and Cowan, 1979].

Repeated infusions of atropine into the medial septum after onset of convulsions resulted in evident anticonvulsant impact, but the seizure activity could not be terminated. Pretreatment with GABAergic modulators or anticholinergics affecting the seizure controlling area tempestas can prevent onset of convulsions in response to soman [Myhrer et al., 2006b; 2008a]. However, repeated infusions of atropine into the area tempestas after onset of soman-induced convulsions do not terminate the seizure activity [Myhrer and Enger, unpublished data]. The potential for focal post-treatment in seizure controlling areas seems to be quite different from pretreatment, because nerve agent-induced seizures appear to make up complex partial seizures progressing to secondarily generalized seizures accompanied by tonic-clonic convulsions [Myhrer, 2007]. The tonic extension of hind limbs probably reflects the most intense seizure activity involving both the forebrain and brainstem [Swinyard, 1973]. Consequently, large parts of the central nervous system must necessarily be affected by anticonvulsants to stop ongoing seizures. Systemic application of drugs exerting cholinergic and glutamatergic antagonism, as well as GABAergic agonism, is required to terminate nerve agent-evoked seizures well after their onset. The combination of procyclidine, diazepam, and pentobarbital or procyclidine and propofol can effectively terminate soman-

induced seizures in rats when administered 30 – 40 min after onset [Myhrer et al., 2003, 2006a]. However, some rats die within 24 h after treatment. The anesthetics used in the above combinations (pentobarbital, propofol) have depressant effects on the respiratory center in the brainstem. For this reason, anticonvulsants affecting the forebrain only would be safer to use. The results from the present and previous studies [Denoyer et al., 1992; Lallement et al., 1992b] suggest that post-treatment, in terms of local microinfusions in the forebrain, can have an anticonvulsant impact. These findings clarify an important principle that after seizure onset, drugs that mainly affect the forebrain probably may terminate seizures.

In summary, microinfusion of anticholinergics into the medial septum assures anticonvulsant efficacy against seizures that are subsequently triggered by soman. Anticonvulsant effects are also achieved by microinfusions after seizure onset, but the impact is far from sufficient to terminate seizures. The medial septum does not appear to be a potent trigger site for seizures, as noted from microinfusion of soman.

## TABLES

Table 1

Anticonvulsant effects of microinfusions (1  $\mu$ L) of drugs into the medial septum of rats intoxicated by soman (1.3 x LD<sub>50</sub>) measured in terms of latency to onset of seizures/convulsions

Latency (min) to seizures/convulsions			
Drug	Dose	N	Mean $\pm$ (S.E.M.)
Saline	-	8	5.9 $\pm$ 0.8
Atropine	100 $\mu$ g	8	12.6 $\pm$ 1.7 <sup>b</sup>
Scopolamine	1 $\mu$ g	8	12.8 $\pm$ 1.6 <sup>b</sup>
Procyclidine	6 $\mu$ g	8	10.8 $\pm$ 0.5 <sup>a</sup>
Ketamine	50 $\mu$ g	8	6.5 $\pm$ 0.8
Muscimol	120 ng	8	6.1 $\pm$ 0.6

Significantly different from saline-treated control group <sup>a</sup> $P$ <0.05, <sup>b</sup> $P$ <0.001  
S.E.M. is the standard error of the mean

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