

## Microinjection of GABAergic agents into the anterior nucleus of the thalamus modulates pilocarpine-induced seizures and status epilepticus

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### ABSTRACT

The anterior nucleus of the thalamus (AN) has been suggested as a potential target for seizure modulation in animal models and patients with refractory epilepsy. We investigate whether microinjections of GABAergic agonists into the AN were protective against pilocarpine-induced generalized seizures and status epilepticus (SE). Rats were treated with bilateral AN injections of muscimol (160 or 80 nmol), bicuculline (15 nmol), or saline (controls) 20 min prior to pilocarpine administration (350 mg/kg i.p.). Electrographic recordings were used to confirm seizure activity. We found that pretreatment with AN muscimol 160 nmol increased the latency to seizures and SE by 2.5–3.0-fold. This dose however was associated with side effects, particularly hypotonia. AN bicuculline was proconvulsant, whereas no major effect was observed after muscimol 80 nmol injections. The percentage of animals that developed SE was similar across groups. Overall, microinjection of high doses of muscimol into the AN delayed the occurrence of pilocarpine-induced seizures and SE but was not able to prevent these events.

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## 1. Introduction

The anterior nucleus of the thalamus (AN) occupies a central position in limbic circuits and has been suggested as a potential target for seizure modulation in animal models and patients with refractory epilepsy.<sup>1–10</sup> In rodents, anticonvulsant effects have been observed with different local AN interventions, including lesions, high frequency stimulation and the microinjection of GABAergic agonists.<sup>2,5–7,10–13</sup> In a previous report, we have compared the effects of AN thalamotomies and stimulation in the pilocarpine model of epilepsy.<sup>2</sup> While lesions were extremely effective against seizures and status epilepticus (SE), stimulation only prolonged the latency to these events.<sup>2</sup> As AN thalamotomies have been associated with significant memory disturbance,<sup>14,15</sup> alternative therapeutic strategies leading to an anticonvulsant outcome are still needed. In the pentylenetetrazol (PTZ) model, the injection of GABAergic agonists into the AN were partially

protective against seizures.<sup>5,6</sup> We investigate whether GABAergic agonists microinjected into the AN are also protective against pilocarpine-induced seizures and SE.

## 2. Methods

### 2.1. Animal groups

Animals were divided into 4 groups: (1) muscimol 160 nmol; (2) muscimol 80 nmol; (3) bicuculline 15 nmol; or (4) saline (controls). These doses were chosen as they have been previously shown to influence seizure activity in the PTZ model.<sup>6</sup> All protocols were approved by the Research Ethics Board of the Federal University of São Paulo (Universidade Federal de São Paulo).

### 2.2. Stereotactic procedures

Adult male Wistar rats (250–300 g) were anesthetized with ketamine/xylazine (100/7.5 mg/kg intraperitoneally; i.p.) and had their heads fixed to a stereotactic apparatus (Model 900, David Kopf Instruments). Stainless steel guide cannulae (21 G; 10 mm of length) were bilaterally implanted into the AN at the following coordinates relative to the bregma: anteroposterior (AP) –1.4,

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mediolateral (ML)  $\pm 1.2$ , and depth 4.8.<sup>16</sup> In addition, 2–3 animals per group were implanted with two stainless steel epidural screws for electrographic recordings (AP +0.4, ML 1.4 and AP –3.0, ML 3.4 to the right).<sup>16</sup> Guide cannulae and epidural electrodes were fixed to the skull with dental acrylic cement. At the end of the procedures, 30 G stainless steel stylets were inserted into the cannulae to avoid obstruction. One concern when drug injections are carried out in the dorsal thalamus is that an eventual extravasation to the lateral ventricle might interfere with outcome. To assess the effects of ventricular muscimol injections, a different group of animals was implanted with ventricular cannulae (AP –0.8, ML 1.4 to the right at a depth of 3.4 mm).<sup>16</sup>

### 2.3. Intraparenchymal drug administration, pilocarpine injections and ictal assessment

Experiments were conducted 5–7 days after surgeries. Animals were initially provided methylscopolamine (1 mg/kg i.p.) to block the undesirable peripheral effects of pilocarpine. Ten minutes later, they received muscimol, bicuculline or saline bilaterally into the AN. Muscimol (160 or 80 nmol; Research Biochemicals International) was dissolved in 0.5 or 0.2  $\mu$ l of a 2% Evans blue saline solution. To dissolve bicuculline (Research Biochemicals International, Natick, MA) glacial acetic acid was used. The solution was titrated to pH 6.0 with 1 NaOH and subsequently brought up to a volume of 0.5  $\mu$ l with 2% Evans blue saline, similar to previous reports.<sup>6</sup> Control animals received 0.5 or 0.2  $\mu$ l of a 2% Evans blue saline solution.

Microinjection of the drugs was carried out with a Hamilton syringe, connected via a silicon tube to a blunt tip 30 G stainless steel needle (11 mm of length). Just before the administration of the drugs, the stylets were removed from the guide cannulae and the 30 G needle inserted in place. As the needle was longer than the cannulae, the last millimeter of its tip protruded to the brain parenchyma. Injections were done over 1 min and the needle was left in place for an additional minute. Thereafter, the needle was removed and the stylet reinserted into the guide cannulae. The procedure was then repeated in the contralateral side.

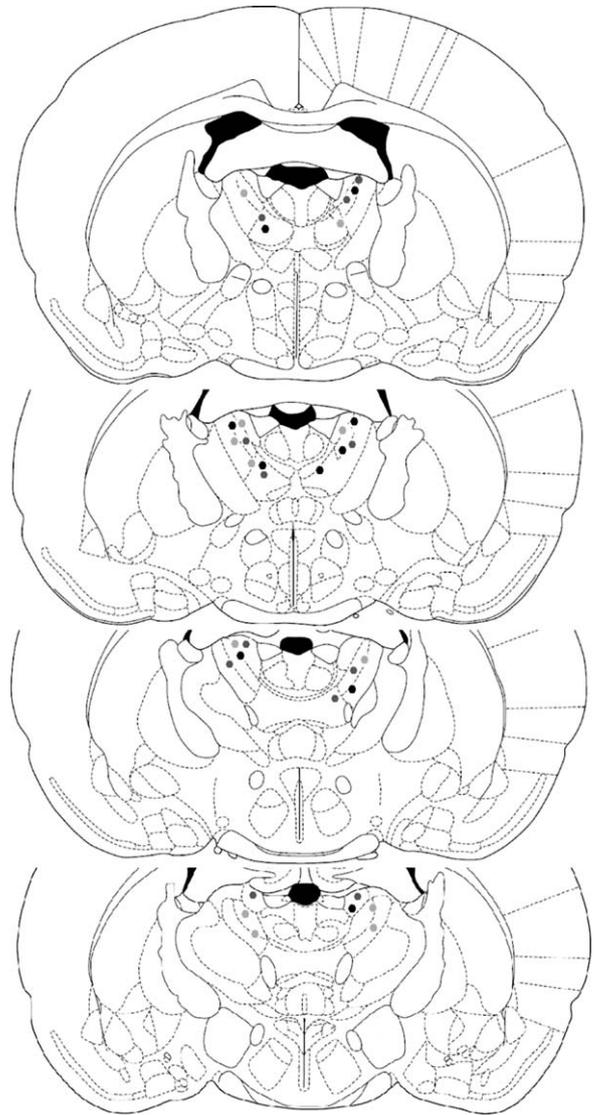
Pilocarpine (350 mg/kg i.p. in a single bolus injection) was given to the animals 30 min after methylscopolamine (20 min after the beginning of the intraparenchymal administration of the drugs). The following variables were assessed: (1) latency to the first behavioral generalized seizure (time elapsed from pilocarpine administration to the first behavioral generalized seizure); (2) latency to SE (time elapsed from pilocarpine administration to the moment when behavioral seizures became uninterrupted); and (3) percentage of animals that developed SE.

After pilocarpine administration commonly observed ictal manifestations include a motionless state, orofacial automatisms, forelimb clonus, and generalized tonic and/or clonic events, which ultimately lead to rearing and falling.<sup>17,18</sup> For the purpose of this study, we have only recorded behavioral seizures characterized by clonic, tonic, or tonic–clonic movements leading to rearing and/or falling.

### 2.4. EEG analysis and histology

EEG analysis was conducted using a bipolar montage (cortical screw 1 vs. 2). Signals were recorded using an Embla unit (Embla N7000, Iceland) from baseline (5 min prior to the pharmacological experiments) to the development of SE. Electrographic analysis was conducted just to corroborate behavioral findings.

To assess the location of the guide cannulae and injection sites, animals were sacrificed by deep anesthesia with thiopental (60 mg/kg i.p.), followed by transcardiac perfusion with 0.9% saline and 10% formaldehyde. Coronal sections with a 24- $\mu$ m thickness were cut on a cryostat, collected in phosphate buffer,



**Fig. 1.** Location of the cannulae used for microinjecting GABAergic agents into the anterior nucleus of the thalamus (AN). Schematic representation of the tips of the cannulae through which 0.5  $\mu$ l of muscimol 160 nmol (●), muscimol 80 nmol (●), or bicuculline (●) were successfully injected within the AN (reprinted from Ref. 16; p. 23–26; Copyright (1998) with permission from Elsevier). Cannulae placement in animals receiving 0.2  $\mu$ l injections was similar to that represented in this figure.

mounted on glass slides, and stained with cresyl violet. Only animals with injections in the AN were considered in this study (Fig. 1). Overall, a total of 68 rats were used, 5 of which ended up with misplaced cannulae.

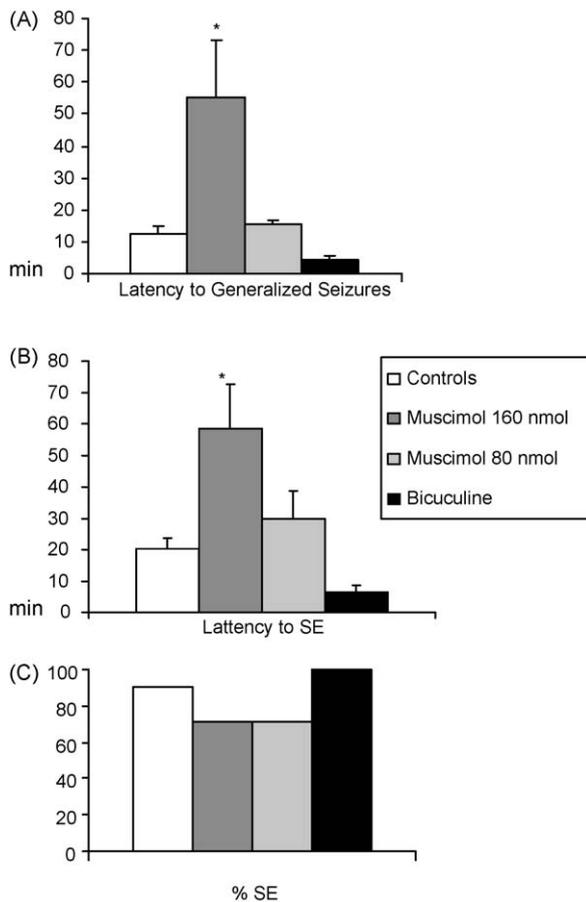
### 2.5. Statistical analysis

One-way ANOVA (post hoc Tukey HSD) and  $\chi^2$  tests were used to compare data across groups. Statistical significance was set at  $p = 0.05$ .  $p$ -Values along the text are post hoc comparisons of experimental animals and controls.

## 3. Results

### 3.1. AN 0.5 $\mu$ l muscimol and bicuculline injections

We found that AN muscimol injections were able to modulate seizure activity induced by acute pilocarpine administration. This was particularly due to the group receiving 160 nmol ( $n = 6$ ), which had a 2–3-fold increase in the latency to seizures and SE ( $p < 0.001$



**Fig. 2.** GABAergic agents microinjected into the anterior nucleus of the thalamus (AN) influence the development of pilocarpine-induced seizures and status epilepticus (SE). (A and B) Animals receiving AN 160 nmol muscimol 0.5  $\mu$ l injections ( $n = 6$ ) had a significantly increased latency to seizures and SE as compared to controls ( $n = 8$ ;  $p < 0.001$  and  $p = 0.01$ ). In contrast, 80 nmol muscimol injections ( $n = 6$ ) were not protective, whereas AN bicuculline ( $n = 5$ ) was proconvulsant. (C) No significant differences were observed in the percentage of animals developing SE across groups. Lines on top of the bars represent standard deviations. \*Statistically significant.

and  $p = 0.01$ ; Fig. 2). In contrast, muscimol 80 nmol injections ( $p = 0.2$  and  $p = 0.2$ ;  $n = 6$ ) were not protective, while AN bicuculline ( $p = 0.1$  and  $p = 0.09$ ;  $n = 5$ ) was proconvulsant (Fig. 2). As all bicuculline treated animals died a few minutes after status, we decided not to pursue further experiments with this drug (e.g. to increase the sample size for reaching statistical significance). Of note, mortality in our study has only occurred after pilocarpine administration. No animal has died before or after thalamic injections.

Rats receiving muscimol 160 nmol and pilocarpine developed side effects approximately 25–30 min after AN injections (5–10 min following pilocarpine administration). These were mainly characterized by a change in posture (spreading the hindlimbs far apart) and some degree of hypotonia. This pattern of adverse events was noticed in all animals receiving muscimol 160 nmol, independent on the site of injections (e.g. anterodorsal (AD), anteroventral (AV) or anteromedial thalamic nuclei (AM)). Of note, side effects were not observed in animals given AN muscimol 160 nmol without pilocarpine ( $n = 3$ ), suggesting that toxicity was likely a consequence of the interaction between treatments.

### 3.2. Animals treated with 0.2 $\mu$ l AN injections and ventricular muscimol

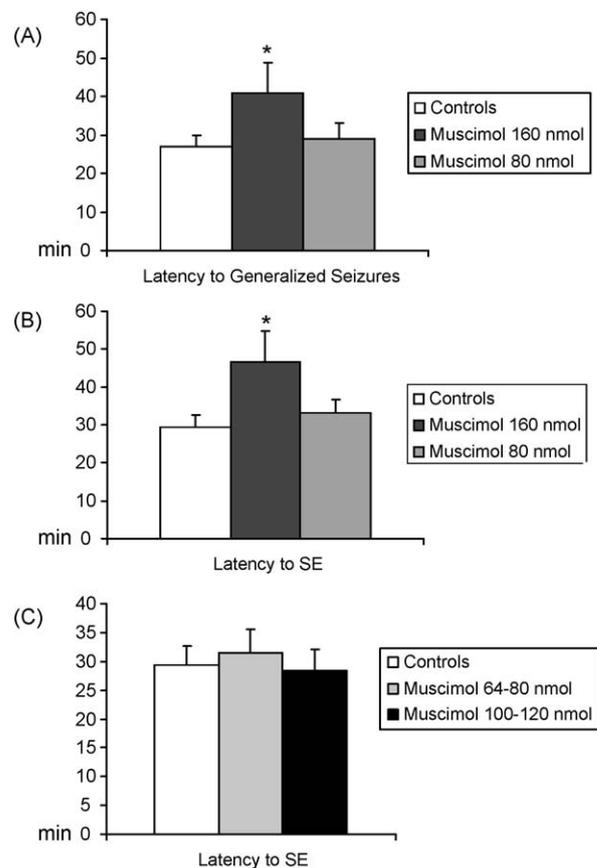
To rule out that outcome after AN muscimol injections was due to an eventual ventricular extravasation of the drug we (1) injected

a group of animals with smaller volumes of AN muscimol and (2) characterized the effects of ventricular muscimol injections.

As in our initial experiments, latency to seizures and SE in animals receiving 0.2  $\mu$ l of muscimol 160 nmol into the AN ( $n = 11$ ) was significantly increased as compared to controls ( $p < 0.01$  and  $p = 0.02$ ;  $n = 10$ ; Fig. 3A and B). Side effects in this group occurred in all animals and were similar to those after 0.5  $\mu$ l injections.

Muscimol ventricular injections (0.2  $\mu$ l) were conducted unilaterally at the following concentrations: 64–80 nmol ( $n = 4$ ), 100–120 nmol ( $n = 6$ ), 160 nmol ( $n = 4$ ). Those were chosen as they would correspond to a hypothetical ventricular extravasation of 20–25, 30–40 or 50% of the bilateral 160 nmol AN injections. Animals given 100–120 nmol of ventricular muscimol with no pilocarpine ( $n = 2$ ) developed a specific pattern of side effects that was different from that observed in AN treated groups. Approximately 10–15 min after the injections, they became slightly hypotonic, moving slowly in circles around the cage. This was followed by a state of decreased reactivity to external stimuli, which lasted a few hours. Once the animals recovered, they developed significant hyperphagia. No adverse effects were observed after 64–80 nmol injections, while 160 nmol of muscimol into the ventricle was associated with severe hypotonia and respiratory arrest in 5 min.

Neither 100–120 nmol ( $n = 4$ ) nor 64–80 nmol of ventricular muscimol ( $n = 4$ ) were protective against pilocarpine-induced SE (Fig. 3C). The pattern of side effects in these animals was similar to that described above.



**Fig. 3.** Latency for pilocarpine-induced seizures and status epilepticus (SE) in animals treated with anterior thalamic nucleus (AN) or ventricular 0.2  $\mu$ l muscimol injections. (A and B) Animals receiving AN 160 nmol muscimol ( $n = 11$ ) had a significantly increased latency to seizures and SE as compared to controls ( $n = 10$ ;  $p < 0.01$  and  $p = 0.02$ ). (C) In contrast, injections of 64–80 nmol ( $n = 4$ ) or 100–120 nmol ( $n = 4$ ) of ventricular muscimol were not protective. Lines on top of the bars represent standard deviations. \*Statistically significant.



**Fig. 4.** Electrographic activity recorded in the cerebral cortex after pilocarpine treatment. EEG tracings showing the onset of a generalized seizure in animals treated with bilateral AN injections of saline (upper trace in A) or muscimol 160 nmol (upper trace in B). In both groups, status epilepticus was characterized by continuous high-voltage fast spiking activity (lower traces in A and B). Vertical scale bar, 100 mV; horizontal scale bar, 5 s.

### 3.3. Electrographic recordings

As previously mentioned, EEG recordings in our study were only carried out to show that the events characterized behaviorally were actual seizures and SE. In saline-treated controls and animals that had AN muscimol 160 or 80 nmol injections, seizures were characterized by repetitive high-voltage fast spiking discharges. SE was characterized by continuous high-voltage fast spiking activity (Fig. 4).

### 3.4. Misplaced injections

Misplaced injections were documented in 5 animals. Three received either muscimol 160 nmol ( $n = 1$ ) or 80 nmol ( $n = 2$ ) in the border of the reticular nucleus and nucleus reuniens. None had an anticonvulsant effect. One animal received 160 nmol of muscimol unilaterally into the AN with the contralateral injection spilling over to the ventricle. It developed severe hypotonia immediately after the injection and died within minutes. The other animal had bicuculline injected in one of the ventricles, developing violent tonic seizures within minutes and dying soon after.

## 4. Discussion

We found that AN muscimol injections were somewhat protective against pilocarpine-induced seizures and SE. This however, was only observed at doses associated with adverse effects.

Our results are in line with previous work in other animal models of epilepsy. In a series of publications, Mirski and colleagues have shown that AN muscimol injections reduced PTZ electrographic seizures, whereas AN  $\gamma$ -vinyl-GABA (inhibitor of GABA transaminase) decreased the number of animals developing clinical seizures.<sup>5,6</sup> In contrast, AN injections of  $\gamma$ -vinyl-GABA were not protective against maximal electroshock-induced seizures.<sup>5</sup> In addition to the AN, the intraparenchymal administration of GABAergic agonists in nearby thalamic targets, including the mediodorsal, reuniens/rhomboid, and anterior intralaminar nuclei, has also been shown to modulate seizure activity in different models of epilepsy.<sup>19–23</sup> Different agents have been shown to induce convulsive states in rodents. We have chosen pilocarpine because, in addition to its well-established validity as a model, it has been used in our former studies to assess the role of AN radiofrequency lesions and DBS against seizures and SE.<sup>2,10</sup> Though the mechanisms of epileptogenesis following pilocarpine are still unclear, the primary sites of action of this cholinergic

compound seem to be cerebral structures containing a high concentration of muscarinic receptors.<sup>24</sup> As the AN is one of such regions,<sup>25,26</sup> it would be conceivable to hypothesize that muscimol injections could disrupt the propagation of ictal activity to limbic circuits. In humans, AN DBS has been suggested to be particularly effective against partial and secondary generalized seizures.<sup>1,4,8,9</sup> Bearing in mind the AN connectivity, future investigation is still needed to clarify whether the efficacy of interventions within this nucleus would improve with the use of models characterized by a more focal induction of limbic seizures (i.e. kindling in the amygdala or intrahippocampal kainic acid injections).

One of the potential drawbacks of studies investigating the effects of intraparenchymal drug administration in the dorsal thalamus is the diffusion of the compounds into adjacent structures, including the ventricles. Though we cannot fully rule out this possibility, we find it to be unlikely for several reasons: (1) a smaller volume of drug injected into the AN led to a similar outcome as compared to 0.5  $\mu$ l injections, (2) the pattern of adverse events in animals receiving muscimol in all subdivisions of the AN was fairly similar (even with the AM being relatively far from the thalamic/ventricular border), (3) side effects in animals receiving ventricular muscimol were different from those in the AN groups, (4) ventricular muscimol injections were not protective against seizures and SE, even at toxic doses. Altogether, these findings suggest that the anticonvulsant effects of AN muscimol in our study were likely due to a local effect of the drug within the thalamus.

Taking into account the considerations above, it is important to discuss potential sites of action of muscimol within the AN. Most cells in this nuclear complex are immunocytochemically positive for glutamate and aspartate.<sup>27</sup> GABAergic neurons comprise a minor population, which has mainly been described in the AD.<sup>27,28</sup> Similarly, GABAergic terminals are primarily found in AD, but also in AV and AM.<sup>27–29</sup> In rats, these are mainly associated with afferent projections from the rostral portion of the thalamic reticular nucleus.<sup>30,31</sup> In this context, one might suggest that muscimol primarily affected AN cells receiving innervation from either the reticular nucleus or local circuit interneurons. Whether this mechanism is actually responsible for the anticonvulsant effects of AN muscimol injections remains to be demonstrated.

We have so far investigated the effects of electrical stimulation, lesions and the topical administration of drugs into the AN of pilocarpine treated rats. Even though a direct comparison across techniques has not been conducted, results obtained after AN 160 nmol muscimol injections were similar in magnitude to those previously described with AN stimulation.<sup>2,10</sup> Muscimol is a GABAergic agonist that leads to a chemical inactivation of cell bodies nearby the injections. In contrast, commonly accepted mechanisms of DBS comprise not only a depolarization block of local neuronal populations but also the activation of axonal pathways nearby the electrodes.<sup>32,33</sup> In this context, one could hypothesize that the functional inactivation of the AN may be an important feature for the increase in latency to pilocarpine-induced seizures and SE after either stimulation or muscimol injections.

In summary, we found that the local administration of high doses of muscimol into the AN delayed the occurrence of pilocarpine-induced seizures and SE. The impact of this treatment in the duration of seizures is still unknown and remains to be elucidated. So far, the only therapy capable of hindering these events in our series of experiments in the pilocarpine model of epilepsy was AN thalamotomy.<sup>2</sup> This treatment however, did not lead to a significant reduction in seizure frequency in chronic epileptic animals<sup>34</sup> and is known to cause significant memory disturbance.<sup>14,15</sup> Additional investigation to characterize suitable treatment modalities to prevent/block seizure activity with limited adverse effects is still necessary.

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