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Short communication

Lack of robust anticonvulsant effects of muscimol microinfusions in the anterior substantia nigra of kindled rats

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Abstract

The substantia nigra pars reticulata is thought to control the spread of seizures in various seizure models. Potentiation of γ-aminobutyrate (GABA)-mediated transmission in this region by intranigral administration of drugs such as muscimol has been shown to inhibit seizure propagation in such models, including the kindling model of epilepsy. More recent studies have shown that the effects on seizures are site-specific within the substantia nigra pars reticulata. Using flurothyl to induce clonic seizures, it was reported that bilateral microinfusions of muscimol into the anterior substantia nigra pars reticulata were anticonvulsant, while similar infusions into the posterior pars reticulata were proconvulsant. This prompted us to reevaluate the effects of intranigral muscimol in the kindling model with particular emphasis on the anterior substantia nigra pars reticulata. In amygdala kindled rats, muscimol was bilaterally infused into the anterior pars reticulata at doses of either 60 or 120 ng. Thirty minutes later, the threshold for induction of afterdischarges in the amygdala and the threshold for generalized seizures were determined in each rat. Furthermore, severity and duration of seizures at threshold currents were recorded. Unexpectedly, muscimol failed to increase seizure thresholds or to significantly reduce seizure severity or duration of motor seizures, although there was a moderate reduction in motor seizure duration in several rats. The data indicate that, in contrast to flurothyl seizures, in kindled rats the anterior pars reticulata of the substantia nigra is not a site at which muscimol causes robust anticonvulsant effects.

Keywords: GABA (γ-aminobutyric acid); Seizure; Epilepsy; Kindling

1. Introduction

Seizure activity does not spread randomly throughout the brain but rather is generated and propagated by specific anatomical routes (Gale, 1985, 1988; Löscher and Ebert, 1996). At least in part, the spread of seizures follows pathways that are also utilized in normal movements. While seizures can be initiated experimentally by a large number of neuronal manipulations (Löscher and Schmidt, 1988), the behavioral alterations associated with different means of seizure induction are often remarkably similar, suggesting that certain propagation pathways may function as common denominators for the development of certain types of epileptic seizures, independent of the specific pathological condition involved in their induction (Gale, 1988).

Numerous data strongly indicate that the substantia nigra pars reticulata serves a gating function in different types of seizures, restricting the propagation of epileptic activity (Gale, 1985, 1988; Löscher and Ebert, 1996). Pharmacological potentiation of γ-aminobutyrate (GABA)-mediated neurotransmission in the pars reticulata, resulting in inhibition of nigral output neurons, is an effective means of suppressing seizure propagation in a wide range of convulsive and non-convulsive seizure types (Gale, 1985, 1988, 1992; Löscher and Ebert, 1996; Depaulis et al., 1994). In the rat kindling model of temporal lobe epilepsy, the most common type of epilepsy, bilateral microinjection of the $GABA_A$ receptor agonist muscimol or the GABA-elevating drug vigabatrin into the substantia nigra pars reticulata has been reported to suppress both motor and limbic seizures induced by stimulation of the amygdala or other limbic structures, indicating that the substantia nigra pars reticulata not only modulates the propagation of seizure activity from rostral to caudal sites but is actively involved in the generation of limbic seizures

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(Le Gal La Salle et al., 1983; McNamara et al., 1984; Löscher et al., 1987).

More recently, Moshé's group reported that the nigral effects on seizures are site-specific within the substantia nigra pars reticulata (Moshé et al., 1994, 1995; Veliskova et al., 1998b). Using flurothyl to induce clonic seizures, the latter group reported that bilateral infusions of muscimol into the anterior pars reticulata were anticonvulsant, while similar infusions into posterior pars reticulata were proconvulsant, indicating that there are two topographically distinct functional regions within the pars reticulata of the substantia nigra. Subsequent studies showed that these regions also differ in the expression of the $GABA_A$ receptor α_1-subunit mRNA (Veliskova et al., 1998a). In a cooperation between Moshé's and our group, it was shown that the α_1-subunit-preferring $GABA_A$/benzodiazepine receptor agonist zolpidem was only anticonvulsant when injected into the anterior but not the posterior pars reticulata (Veliskova et al., 1998b). Based on these findings in the flurothyl seizure model, we reexamined the effects of intranigral muscimol in the amygdala kindling model and studied whether microinfusions of muscimol into the anterior pars reticulata are an effective means of increasing seizure threshold and/or decreasing seizure propagation.

2. Materials and methods

Female Wistar rats (Harlan–Winkelmann; Borchen, Germany; body weight 200–220 g) were housed individually and kept under controlled environmental conditions with a 12/12 h light/dark cycle, light on at 7:00 a.m., for at least 1 week before the experiments. Standard laboratory chow (Altromin 1324 standard diet) and tap water were allowed ad libitum. All experiments were done in compliance with the German Animal Welfare Act.

A total of 13 rats were anesthetized with chloral hydrate (360 mg/kg i.p.) and a Teflon-isolated bipolar stainless steel electrode was stereotaxically implanted for stimulation of the right basolateral amygdala and recording of afterdischarges. The stereotaxic coordinates in millimeter relative to bregma according to the atlas of Paxinos and Watson (1998) were: posterior (P) −2.8, lateral (L) 4.8, and ventral (V) 8.6 mm. One screw, placed above the left parietal cortex, served as the indifferent reference electrode. In addition to the amygdala electrode, the anesthetized rats received guide cannulae (outer diameter 640 μm, inner diameter 400 μm) bilaterally into one of the following locations in the substantia nigra pars reticulata: anterior pars reticulata, P −5.0, L ±2.2, V 7.0; posterior pars reticulata, P −5.8, L ±2.2, V 7.0. The guide cannulae were positioned 1 mm above the intended injection site and closed by removable dummy cannulae (obturators) of the same length before and between drug experiments. Additional skull screws and dental acrylic cement anchored the entire headset. After surgery, the animals were allowed a recovery period of 2 weeks. The rats were then electrically kindled via the electrode in the basolateral amygdala.

For kindling, the rats were stimulated once daily with a suprathreshold current of 500 μA until 10 stage 5 seizures were elicited. Severity of seizures was classified according to Racine (1972): stage 1, immobility and facial automatisms (eye closure, facial clonus); stage 2, head nodding, associated with more severe facial clonus; stage 3, unilateral forelimb clonus; stage 4, rearing and bilateral forelimb clonus; stage 5, rearing and falling accompanied by generalized tonic–clonic seizures. In addition to seizure severity, seizure duration and afterdischarge duration were determined. Seizure duration was further subdivided into seizure duration-1 and seizure duration-2. Seizure duration-1 was the period of limbic (stages 1–3) and/or motor seizures (stages 4/5), while seizure duration-2 was seizure duration-1 plus the time of postictal immobility (often associated with signs of focal seizure activity) until the rats showed normal behavior and reactions. Afterdischarge duration was the total time of spikes in the electroencephalogram, including the time of stimulation.

In fully kindled rats, the threshold for inducing afterdischarges was determined by an ascending stairstep procedure, starting at an initial current intensity of 25 μA (1 s train, 1 ms stimulus at 50 Hz), followed by increases in current intensity by about 20% of the previous current at intervals of 1 min until the stimulation induced afterdischarges of at least 3 s duration. Fully kindled rats show either partial (stages 1–3) or generalized (stages 4 or 5) seizures at the afterdischarge threshold. In rats which showed only a partial behavioral seizure at the afterdischarge threshold, the current intensity was continuously increased in steps as described above until a generalized seizure was elicited (generalized seizure threshold), whereas in rats showing a generalized seizure at the afterdischarge threshold, afterdischarge threshold was equal to generalized seizure threshold. Seizure severity, seizure duration, and afterdischarge duration were recorded at afterdischarge threshold and generalized seizure threshold currents as described above. Per definition, seizure duration-1 at generalized seizure threshold currents was the duration of motor (stage 4 or 5) seizures, used as parameter for testing of intranigral muscimol in a previous study by McNamara et al. (1984), so that this parameter allowed a direct comparison between studies. Afterdischarge threshold, generalized seizure threshold, and seizure parameters were determined twice a week to establish reliable control thresholds prior to microinjection. The threshold determinations before and after microinjection were performed at the same days of the week to avoid interday variance and at the same time of the day (starting 9:00 a.m.) to avoid intraday variance in seizure thresholds between animals.

For bilateral microinjection of muscimol (60 or 120 ng) or vehicle into the substantia nigra pars reticulata, the obturators were removed and injection cannulae (outer

diameter 350 μm, inner diameter 150 μm) were inserted in the bilaterally implanted guide cannulae. The injection cannula was 1 mm longer than the guide cannula to reach the injection site within the substantia nigra pars reticulata. The injection cannulae were connected to a 0.5-μl Hamilton syringe, respectively, by about 30 cm of flexible tubing which allowed the infusion of drugs into freely moving animals in an observation cage. One minute after inserting the injection cannulae, 60 or 120 ng muscimol in 250 nl saline was infused per hemisphere over a period of 4 min. The infusion volume was controlled by the movement of a small air bubble in the tubing. The injection cannulae were left in place for 1 min after the end of injection. Afterdischarge threshold, generalized seizure threshold, and seizure parameters were determined 30 min after microinjection. The regular determination of seizure thresholds and seizure parameters was continued twice weekly for at least four times. After the thresholds were found to be stable again, the next microinjection experiment was done. In all animals, the following sequence of drug injection was kept: 60 ng muscimol, vehicle (saline), and 120 ng muscimol. All rats were observed for behavioral side effects (stereotyped behavior, altered body and head posture) during and after the microinjection until animals reacted normally again.

Muscimol hydrobromide was purchased from Sigma-Aldrich (Taufkirchen, Germany) and dissolved in saline to obtain 60 or 120 ng of the free base in the injection volume of 250 nl. Aliquots of 1 ml solution were kept at −20 °C. The pH of the solutions and vehicle alone were adjusted to 7.3 to 7.4.

After termination of the experiments, the location of injection and stimulation sites were verified histologically. The rats were deeply anesthetized with chloral hydrate and perfused transcardially with 50 ml saline in 0.01 M phosphate buffer (pH 7.4), followed by 250 ml 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and stored overnight at 4 °C in 30% sucrose in 0.1 M phosphate buffer. Two series of 52 μm coronal sections were cut on a freezing microtome. One series was Nissl-stained with thionine for verification of injection and stimulation sites.

Only animals with correct bilateral location of injection sites within the substantia nigra pars reticulata and stimulation electrode within the basolateral amygdala were used for evaluation of data. For the purpose of comparing injection sites between anterior and posterior pars reticulata, the anterior/posterior division was made between sections −5.3 and −5.6 (according to Shebab et al., 1996). Since the major effect of intranigral muscimol injection reported previously was on motor (stage 4 or 5) seizures (McNamara et al., 1984), only seizure parameters (seizure severity, seizure duration, afterdischarge duration) recorded at generalized seizure threshold were illustrated here. Differences in seizure thresholds, seizure duration and afterdischarge duration before and after microinjection were calculated by paired Student's *t*-test, while the Wilcoxon signed rank test was used in case of seizure severity. All tests were used two-tailed and a $P < 0.05$ was considered significant.

3. Results

Six rats had a bilateral location of injection sites within the anterior substantia nigra pars reticulata (i.e., at −4.8, −5.2, or −5.3 mm from bregma in Fig. 1) and could thus be used for final evaluation of data for the anterior pars reticulata. Microinjection of muscimol at these sites induced typical stereotyped behavioral alterations, consisting of circling, gnawing, sniffing, and, in some rats, inclined position of head and body, which was not seen after microinjection of saline. The behavioral alterations started 4–20 min (mean 12 min) after intranigral application of muscimol and were seen for at least 2 h (in most rats 3–5.5 h).

Bilateral microinjection of vehicle (saline) did not significantly alter afterdischarge threshold (Fig. 2) or seizure parameters recorded at afterdischarge threshold. Since all rats exhibited stage 5 seizures at afterdischarge threshold currents, it was not necessary to determine the generalized seizure threshold separately in these vehicle control experiments so that all seizure parameters shown for generalized

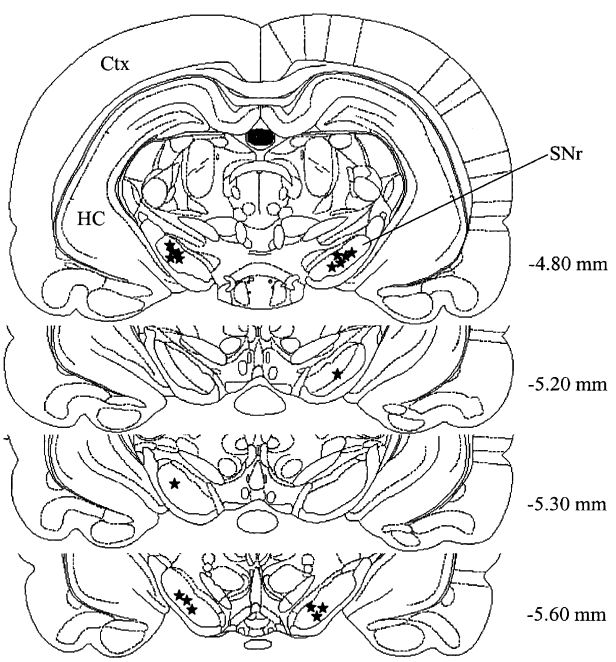

Fig. 1. Schematic reconstruction of the injection sites of muscimol in the substantia nigra pars reticulata of the rat according to the atlas of Paxinos and Watson (1998). All rats received bilateral injections. Data for nine kindled rats with correct bilateral location of injection sites in either anterior (−4.8, −5.2, or −5.3 mm posterior to bregma; $n = 6$) or posterior (−5.6 mm) substantia nigra pars reticulata are shown. Abbreviations: Ctx, cortex; HC, hippocampus; SNr, substantia nigra pars reticulata.

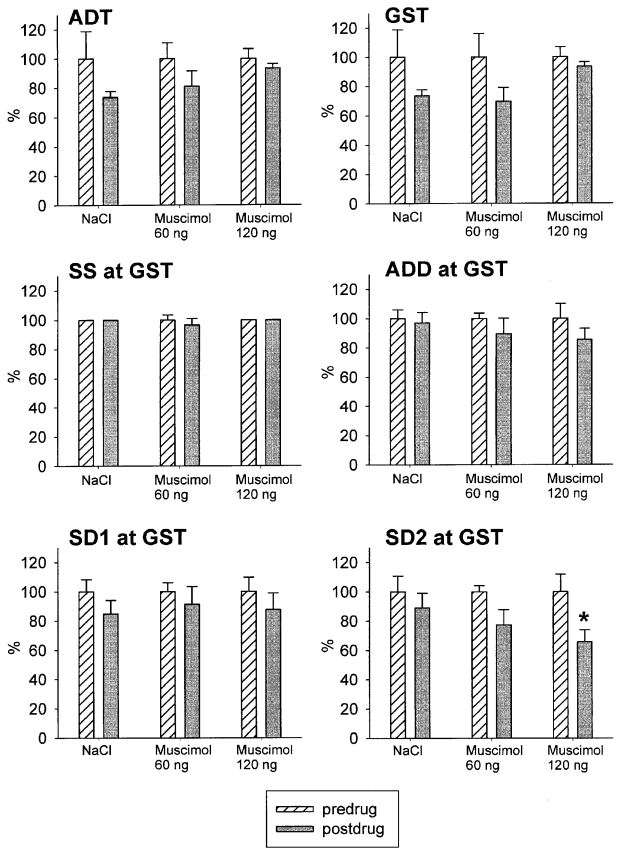

Fig. 2. Effects of muscimol on kindled seizure parameters. Muscimol was bilaterally microinfused into the anterior substantia nigra pars reticulata at either 60 or 120 ng. Thirty minutes after microinfusion, the afterdischarge threshold (ADT) and, in case that only focal seizures occurred at afterdischarge threshold currents, the generalized seizure threshold (GST) were determined, and seizure parameters (seizure severity [SS], afterdischarge duration [ADD], seizure duration-1 [SD1], seizure duration-2 [SD2]) at afterdischarge threshold or generalized seizure threshold currents were recorded. The figure shows afterdischarge threshold and generalized seizure threshold as well as seizure parameters recorded at generalized seizure threshold. All data are means ± S.E. of six fully kindled rats and are shown as percent of predrug control data determined in the same rats 3 to 4 days before the microinfusions of muscimol. Absence of S.E. bars indicate that all rats had the same recordings. To test for the effect of microinjection alone, a vehicle (NaCl) control experiment was done with seizure threshold determination 30 min after microinfusion of vehicle into the anterior substantia nigra pars reticulata. Significant differences from predrug values are shown by asterisk ($P = 0.0073$). Average control data (without microinjection) from the three predrug/prevehicle control experiments were as follows: afterdischarge threshold, 55 ± 5.7 μA; generalized seizure threshold, 64 ± 7.3 μA; seizure severity, 4.9 ± 0.06 (score); afterdischarge duration, 87 ± 5.0 s; seizure duration-1, 68 ± 3.9 s; seizure duration-2, 386 ± 31 s.

seizure threshold in Fig. 2 were the same for afterdischarge threshold.

Bilateral microinjection of muscimol at 60 or 120 ng into the anterior pars reticulata did not significantly increase afterdischarge threshold or generalized seizure threshold (Fig. 2). At the lower dose, some of the rats exhibited only focal seizures at afterdischarge threshold (2/6 in the preinjection experiment and 1/6 in the muscimol experiment), so that the generalized seizure threshold had to be determined in addition to afterdischarge threshold in these rats. Seizure severity at generalized seizure threshold was not affected by 60 ng muscimol in any rat (Fig. 2). In the experiment with 120 ng of muscimol, all rats exhibited stage 5 seizures at afterdischarge threshold currents both on the pretreatment and treatment days. In other words, muscimol did not reduce seizure severity in any of the rats at 120 ng. The duration of motor (stage 4 or 5) seizures (seizure duration-1) was reduced in five of the six rats by muscimol at both doses, but one rat in each experiment exhibited an increase in seizure duration, so that the difference to the predrug control became not significant for the whole group (Fig. 2). When only the 5 rats showing a decrease in motor seizure duration were used for group calculations, the decrease in the experiment with 60 ng of muscimol was significant (seizure duration-1: 50.4 ± 5.4 s after muscimol vs. 62.2 ± 4.4 s on predrug day; $P = 0.0305$), whereas the decrease in the experiment with 120 ng of muscimol was not significant (58.2 ± 9.1 vs. 68.2 ± 7.1 s; $P = 0.078$). The overall seizure duration (seizure duration-2) in the whole group ($n = 6$) was significantly reduced by muscimol at 120 (33% difference to predrug control) but not 60 ng (Fig. 2). Afterdischarge duration recorded at generalized seizure threshold tended to be decreased by both doses of muscimol, but the difference to predrug control was not significant (Fig. 2).

Because of the unexpected lack of any robust anticonvulsant effect of muscimol after microinjection into the anterior substantia nigra pars reticulata of kindled rats, some additional rats were injected into the posterior pars reticulata. Three of these rats had a correct bilateral location of injection sites (at −5.6 in Fig. 1), so that data from these rats could be used for comparison with results from anterior injection of muscimol. At 60 ng muscimol, none of the three rats showed an increase in afterdischarge threshold or generalized seizure threshold, while at 120 ng one of three rats exhibited an increase in afterdischarge threshold and generalized seizure threshold (not illustrated). Seizure severity at generalized seizure threshold was not decreased in any animal by either 60 or 120 ng of muscimol, but one rat which had a stage 3 seizure at afterdischarge threshold during the predrug trial showed no behavioral seizure at afterdischarge threshold following 60 ng muscimol. Further increase of current in this rat then resulted in a stage 5 seizure at the same current which had induced a stage 5 seizure in the predrug trial. Motor seizure duration at generalized seizure threshold was decreased in all three rats at both doses by an average of 18% (60 ng) and 19% (120 ng), respectively. Similar average decreases (18% and 10%) were seen for afterdischarge duration. As with the anterior pars reticulata, the most marked effect of muscimol was seen in terms of a decrease of seizure duration-2, with average reductions of 42% (60 ng) and 58% (120 ng), respectively.

When data from all nine rats with intranigral injections sites (irrespective of anterior or posterior location) were combined, there was a significant reduction of duration of motor seizures at generalized seizure threshold (seizure duration-1) at the higher dose of muscimol (68.2 ± 4.3 vs. 58.1 ± 4.9 s; $P = 0.0066$). Furthermore, seizure duration-2 was significantly decreased at the higher dose of muscimol (not illustrated). Afterdischarge threshold and generalized seizure threshold or seizure severity and afterdischarge duration recorded at afterdischarge threshold or generalized seizure threshold were not significantly affected by microinjection of muscimol in the nine rats.

4. Discussion

To our knowledge, there is only one previous study on the effect of intranigral muscimol on kindled seizures (McNamara et al., 1984). In this study, McNamara et al. (1984) reported that bilateral microinjection of 50 ng muscimol into the substantia nigra pars reticulata abolished motor seizures in seven of eight amygdala kindled rats when animals were stimulated with a current exceeding the generalized seizure threshold by 10%. The interval between injection and amygdala stimulation was 30 min. There was also a 85% suppression of afterdischarge duration by muscimol at this stimulus strength (McNamara et al., 1984). In further experiments, McNamara et al. (1984) found that the inhibitory effect of muscimol on motor seizures was related to an increase in generalized seizure threshold by about 70%. The exact anterior–posterior location of injection sites within the pars reticulata was not described in the study of McNamara et al. (1984). In the present study, in which afterdischarge threshold and generalized seizure threshold were determined in each rat 30 min after bilateral intranigral microinjection of muscimol at doses of either 60 or 120 ng, no significant effects on afterdischarge threshold or generalized seizure threshold were found when microinjections were located in the anterior pars reticulata. Several rats showed a reduction in the duration of motor seizures, but the magnitude of this effect was much smaller compared to the data reported by McNamara et al. (1984). The only significant effect was a reduction in the overall seizure duration (seizure duration-2).

Based on previous data from flurothyl seizures (Moshé et al., 1994, 1995; Veliskova et al., 1998b), we thought that microinjections into the anterior part of the substantia nigra pars reticulata would be particularly effective in inhibiting kindled seizures. Unexpectedly, this was not the case which prompted us to inject muscimol in some rats into the posterior part of the pars reticulata. Again, no marked anticonvulsant effects on motor seizures were seen. When rats with muscimol injections into the anterior or posterior pars reticulata were combined into one group, duration of motor seizures recorded at generalized seizure threshold was significantly reduced by 120 ng muscimol, which would be in line with the data from McNamara et al. (1984), but the effect was much smaller (15%) compared to the 91% suppression reported by the latter group.

Because the aim of the present study was to examine whether the anterior part of the substantia nigra pars reticulata is particularly effective in mediating anticonvulsant effects of intranigral muscimol in kindled rats, we injected only few rats into the posterior pars reticulata, so that our experiments do not exclude that microinjections of muscimol in more posterior parts of the pars reticulata would have been more effective to suppress kindled seizures in larger groups of rats. In this respect, it is important to note that Shehab et al. (1996) have shown that posterior (caudal) rather than anterior (rostral) sites in the pars reticulata are sensitive for suppression of tonic hindlimb extension by muscimol in the maximal electroshock seizure test in rats. The latter authors suggested that the rat SN may contain a functional organization based on a form of somatomotor topography, which is in line with suggestions from Moshé's group (Moshé et al., 1995). However, whereas muscimol's effects on flurothyl seizures were mediated by the anterior pars reticulata (Moshé et al., 1994), muscimol's effects on maximal electroshock seizures were mediated by posterior pars reticulata (Shebab et al., 1996), suggesting that the role of the substantia nigra pars reticulata as a gating mechanism in different seizure models is more complex than previously thought. The present data from the kindling model add to this complexity.

Most data on anticonvulsant effects of intranigral muscimol stem from seizure models such as the maximal electroshock seizure test in which seizures are elicited in normal, healthy rats (Gale, 1985, 1988; Löscher and Ebert, 1996). In contrast, kindling of rats results in chronic brain dysfunctions with diverse neurochemical, neurophysiological and morphological changes in brain regions, including the substantia nigra pars reticulata (Sato et al., 1990; Löscher, 1993; McNamara, 1995). We have previously reported that synaptosomal GABA levels, activities of the GABA synthesizing enzyme glutamate decarboxylase, and GABA receptor binding are significantly decreased in the substantia nigra of amygdala kindled Wistar rats (Löscher and Schwark, 1987), i.e., the rat strain also used for the present study. Thus, one explanation for the lack of robust anticonvulsant effects of intranigral muscimol in the present study could be a decrease in drug targets, i.e., $GABA_A$ receptors, in the substantia nigra pars reticulata of the kindled rats. However, the stereotyped behavioral alterations observed after intranigral injection of muscimol in kindled rats were similar to those reported for non-kindled rats (Shehab et al., 1996), which seems to argue against the possibility that the lack of any marked anticonvulsant effect of muscimol was related to impaired GABAergic neurotransmission in the substantia nigra pars reticulata of kindled rats.

Another explanation for the lack of any robust anticonvulsant effect of intranigral muscimol in kindled Wistar rats is that GABAergic transmission in the substantia nigra pars reticulata is less important for regulation of kindled seizures than previously thought. In line with this possibility, selective bilateral destruction of the anterior and posterior pars reticulata by intranigral injection of ibotenate had no effect on seizure susceptibility, seizure severity or seizure duration in Wistar rats kindled from different limbic sites, including the amygdala (Wahnschaffe and Löscher, 1990). On the other hand, intranigral microinfusion of vigabatrin or transplantation of fetal GABAergic neurons into the substantia nigra pars reticulata exerted anticonvulsant effects in fully kindled Wistar rats (Löscher et al., 1987, 1998), suggesting that the lack of marked effects of intranigral muscimol in this rat strain is drug-specific rather than related to a general inefficiency of GABAergic transmission in the pars reticulata to modulate kindled seizures.

Most previous studies on anticonvulsant effects of intranigral muscimol used male Sprague–Dawley rats (e.g., Gale, 1985; Zhang et al., 1991; Moshé et al., 1995; Shehab et al., 1996), whereas we used female Wistar rats. To our knowledge, it is not known whether gender or strain affect the effects of intranigral muscimol. Intranigral injection of vigabatrin exerts a similar anticonvulsant effect on kindled seizures in Sprague–Dawley and Wistar rats (Le Gal La Salle et al., 1983; McNamara et al., 1984; Löscher et al., 1987). With respect to gender, the oestrous cycle does not affect amygdala-kindled seizures or drug effects on seizures in Wistar rats (Wahnschaffe and Löscher, 1992; Rundfeldt et al., 1990), so that it is unlikely that the low anticonvulsant efficacy of muscimol in the present experiments was related to the sex of the rats.

In conclusion, in contrast to previous data from the flurothyl seizure model (Moshé et al., 1994), microinfusion of muscimol into the anterior substantia nigra pars reticulata does not exert any marked anticonvulsant effects in the kindling model. The pathways responsible for the regulation of seizure activity from pars reticulata are not completely defined (Shehab et al., 1996). It appears that, acting via its several efferent pathways, the substantia nigra pars reticulata can play different roles in regulating propagation of different types of experimental seizures (Zhang et al., 1991). Which of these pathways are involved in nigral effects on kindled seizures remains to be defined.

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