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The effects of central administration of physostigmine in two models of anxiety

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Abstract

The effects of intracerebroventricular and intraseptal (the medial septum) administration of a prototypical acetylcholinesterase inhibitor (AChE-I), physostigmine, and a classic benzodiazepine midazolam on rat behavior in the open field test of neophobia and in the conditioned fear test (freezing reaction) were examined in rats. In the open field test of neophobia midazolam and physostigmine increased at a limited dose range, rat exploratory activity, after intracerebroventricular injection. Physostigmine produced in addition the hyperlocomotory effect. Following intraseptal injections, only physostigmine selectively prolonged the time spent by animals in the central sector of the open field. In the model of a conditioned fear, both midazolam and physostigmine inhibited rat freezing reaction to the aversively conditioned context after intracerebroventricular, but not after intraseptal, pretrial drug administration. The presented data support the notion about the selective anxiolytic-like effects of some AChE-Is. It appears, therefore, that the calming and sedative effects of AChE-Is observed in patients with Alzheimer's disease may be directly related to their anxiolytic action, independent of an improvement in cognitive functions, which in turn may decrease disorientation-induced distress and anxiety.

Keywords: Physostigmine; Midazolam; Intracerebroventricular injections; Intraseptal injections; Neophobia; Contextual fear; Rats

1. Introduction

It has been recently suggested that acetylcholinesterase inhibitors (AChE-Is), represented by a prototypical drug physostigmine, may exert, besides their procognitive action, also anxiolytic-like effects in the rat model of neophobia (Sienkiewicz-Jarosz et al., 2000). This finding concurs with the clinical reports on the calming and sedative actions of AChE-Is in patients with Alzheimer's disease (Levy et al., 1999). However, it is not clear whether such effects of AChE-Is are directly related to their anxiolytic action or are secondary to an improvement in cognition, which may decrease disorientation-induced distress and anxiety. This important topic, related to the central action of AChE-Is, has not been followed in a systematic way by basic research. The research in this area focused on elucidating the role that ligands of the muscarinic and nicotinic receptors may play in controlling animal behavior in anxiety models (File et al., 2000a,b). The anxiolytic-like potential of nicotine and some nicotinic receptor ligands was found to be similar to that of benzodiazepine derivatives (Sienkiewicz-Jarosz et al., 2000). AChE-Is stimulate central cholinergic system in a more balanced way; therefore, they are likely to be devoid of some side effects accompanying the action of direct nicotinic receptor ligands (e.g., the abuse potential of nicotine).

The aim of the present paper was to examine in two models of anxiety the effects of intracerebroventricular and intraseptal (the medial septum) administration of a prototypical AChE-I, physostigmine, and as a control drug a full agonist at the benzodiazepine receptors, midazolam. Intracerebroventricular route of drug administration was chosen

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to minimize the contribution of peripherally induced changes to the central effects of AChE-I. Intraseptal route of drug administration was selected on the basis of numerous drug injection, immunocytochemical (c-Fos gene expression), and lesion studies showing that the septum plays an important role in the control of anxiety (Bitran et al., 1999; Campeau et al., 1997; Cheeta et al., 2000a,b; Degroot et al., 2001; Degroot and Parent, 2001; Duncan et al., 1996; File et al., 2000a; Kask et al., 2001; Lamprea et al., 2000; Pesold and Treit, 1992, 1996; Zhu and McNaughton, 1995). The hippocampal cholinergic system is represented mainly by the septo-hippocampal cholinergic pathway and has been shown to be essential to all the cognitive and emotional functions related to the hippocampus. Cholinergic neuronal bodies are localized in the medial septum and in the diagonal band of Broca, and project their axons towards all areas of the hippocampus proper (CA3, CA1, and dentate gyrus) (Aloisi, 1997; Nicoll, 1985; Wainer et al., 1985).

Two animal models of anxiety were used in the study: (i) neophobia-induced suppression of animals' exploratory activity (Treit and Fundytus, 1989), reflecting the symptoms of patient's anxiety resulting from exposition to a novel environment and (ii) contextual fear conditioning (the freezing reaction of animals exposed to the environment previously paired with the aversive stimulation). It is noteworthy that the model of conditioned fear bears considerable construct and face similarity to the human emotional behavior (Szyndler et al., 2000) and is frequently used in the preclinical studies on the emotional processes.

2. Materials and methods

2.1. Animals

Male Wistar rats, supplied by a licensed breeder (the Górzkowska's farm), weighing 200 ± 20 g at the beginning of the experiment were used in the study. Animals were housed in standard laboratory conditions under a 12-h cycle (lights on at 6:00 a.m.) in a constant temperature (21 ± 2 °C) and 70% humidity. After surgery, the animals were kept individually in plastic cages (30 × 20 × 15 cm) and were gently handled 5 min daily for 5 days before testing. Each experimental and control group consisted of six to nine animals. The experiments were performed between 10:00 a.m. and 6:00 p.m. All experimental procedures using animal subjects were approved by the Committee for Animal Care and Use at the Medical University in Warsaw.

2.2. Drugs

The following drugs were used in the experiments: physostigmine hemisulfate (Sigma-Aldrich, Poland) and midazolam maleate (Hoffman-La Roche, Paris). The drugs were dissolved in saline.

2.3. Intracerebral injections

2.3.1. Surgery

The rats were anaesthetised with ketamine (100 mg/kg ip) and fixed on a stereotaxic apparatus (Stoelting, USA). For intracerebroventricular injections, a 10-mm-long, 22-gauge stainless steel guide cannula was implanted 2.0 mm above the right lateral ventricle according to the rat brain atlas (3.2 mm posteriorly to the bregma, 1.5 mm laterally to the sagittal suture, 2.2 mm below the dura) (Pellegrino et al., 1967). For intraseptal injections, a 26-gauge stainless steel guide cannula (Plastics One) was implanted 1.5 mm above the medial septum according to the rat brain atlas (0.7 mm anteriorly to the bregma, 0.0 mm laterally to the sagittal suture, 5.0 mm below the dura) (Pellegrino et al., 1967). The guide cannula was fixed to the skull with jewellery screws and dental acrylic cement, and the potency of the cannula was maintained by the insertion of a stylet. Seven days after the surgery, rats were subjected to behavioral testing.

2.3.2. Drug administration

Microinjections were given unilaterally using a Hamilton microsyringe connected via polyethylene tubing with an injection needle (24-gauge for intracerebroventricular injections, 28-gauge for intraseptal injections). The drugs were dissolved in saline and injected in a volume of 5 μl at the rate of 1 μl/12 s (intracerebroventricular injections) or in the volume of 0.5 μl at the rate of 0.1 μl/12 s (intraseptal injections); the injection needle was removed after 30 s and the stylet was replaced. The behavioral tests were started 10 min after drug administration. Control groups received appropriate volume of saline. Each animal received two microinjections: first in the open field and then in the contextual fear test, separated by a 7-day-long interval. A separate group of animals was used in the control experiment: the flinch–jump test.

2.4. Histological analysis

After the experiments, the implanted animals were sacrificed. The brains were removed and stored in 5% formaldehyde solution. The frozen tissue was dissected into the slices to establish the place of microinjection. Only the data from animals with the injection site located in the lateral ventricle and septal region were taken into consideration.

2.5. Behavioral tests

2.5.1. Open field test

Ten minutes after saline or drug microinjection, the open field test was performed in a soundproof chamber under dim light and continuous white noise (65 dB) without previous habituation. The open field apparatus consisted of two

round arenas (80-cm diameter) with 30-cm-high walls. The animals were placed close to the wall and the experiment was started immediately afterwards. During 20 min of observation, locomotor activity, the number of central entries, and the time spent in the central sector of the open field (50-cm diameter) were recorded and analyzed with the PC-based Videomot System (TSE, Bad Homburg, Germany). The parameter of thigmotaxis was calculated as a ratio of the number of entries into the central part of testing arena to the rat locomotor activity multiplied by 10,000. The higher the value of the score, the lower the thigmotaxis and the more pronounced the anxiolytic-like effect (Treit and Fundytus, 1989).

2.5.2. Contextual fear-conditioning test

The test was done in two boxes (30 × 30 × 60 cm each) made of Plexiglas, with a grid floor made of stainless steel bars wired to a shock generator. The boxes were cleaned after each trial with 95% ethanol. The experiment was performed during three consecutive days in the same testing boxes and experimental chamber. On the first day, the animals were adapted to the experimental conditions by being placed separately for 2 min in a training box. The following day, 10 min after saline or drug microinjection, the animals were placed in the experimental box and were observed and videotaped for 5 min via a short-circuit television for spontaneously occurring freezing behavior (baseline freezing). Immediately afterwards, the animals received three 0.5-s footshocks (trains of stimuli: 0.7 mA, 150/300 ms, repeated every 60 s). The animals were removed from the testing boxes 3 min after the last shock was delivered. On the following day, the freezing behavior of rats was examined for 10 min in the same experimental chamber and testing boxes. The conditioned response was recorded with the help of a video camera for a later analysis of the freezing reaction. The freezing behavior was defined as the absence of any visible body movements except for those required for respiration. The behavioral observation was performed by an experimenter unaware of the group membership.

2.6. Flinch–jump test

Ten minutes after saline or drug microinjection, the test was performed in the footshock boxes used in the part of the experiment on contextual fear conditioning. The naive rats were placed individually into the box. Shocks were delivered to the grid floor of the test box through a shock generator. After a 3-min period of habituation to the test box, shocks titrations were continued upwards and downwards in a stepwise manner (0.05 mA, 0.05- to 0.85-mA range) depending upon the responsiveness of the rat. The flinch threshold was defined as the lowest shock intensity that elicited any detectable response. The jump threshold was defined as the lowest shock intensity that elicited simultaneous removal of at least three paws (both hindpaws) from the grid. To avoid foot damage, the cutoff of 1.0 mA was established. In this way, the flinch and jump thresholds in milliamperes were defined for each rat. The time gap between shocks was 10 s, and each animal was tested only once. The time between drug administration and testing was the same as in the contextual fear-conditioning test.

2.7. Data analysis

The data are shown as means ± S.E.M. The data involving one control and one treated group were analyzed using Student's *t* test for independent samples. The data involving multiple comparisons were calculated with one-way ANOVA, followed by post hoc LSD test. The confidence limit of $P < .05$ was considered as statistically significant.

3. Results

3.1. Open field

3.1.1. Intracerebroventricular injections

Midazolam administered into lateral ventricle significantly increased time spent in the central sector of the open field

Table 1
The effect of midazolam and physostigmine administered intracerebroventricularly on rat behavior in the open field test

Drug (μg icv)	*n*	Motor activity	Number of central entries	Time in the central sector (s)	Antithigmotactic effect
Midazolam					
0	8	3656.83 ± 572.79	4.50 ± 1.27	9.52 ± 2.92	110.01 ± 28.57
0.1	9	4016.69 ± 232.53	14.33 ± 4.11	55.99 ± 15.86 **	333.35 ± 89.18
20	8	3600.98 ± 231.18	8.25 ± 2.70	9.39 ± 7.73	251.99 ± 82.46
Physostigmine					
0	10	3904.00 ± 329.31	8.70 ± 1.67	11.02 ± 2.38	103.07 ± 16.78
5	9	5897.78 ± 626.63 **	18.00 ± 2.40 **	18.50 ± 3.09 *	155.95 ± 17.98
10	10	5017.78 ± 563.64	12.90 ± 2.81	12.07 ± 2.58	114.41 ± 17.72
20	10	3692.00 ± 288.20	6.89 ± 1.56	4.51 ± 1.22	97.95 ± 21.22

The data are shown as means ± S. E. M. *n* = number of rats.
* $P < .05$, differs from control group.
** $P < .01$.

[$F(2,22)=4.68$, $P<.05$]. The drug produced a tendency to increase the number of central entries [$F(2,22)=2.66$, $P=.09$] and to decrease thigmotaxis [$F(2,22)=2.35$, $P=.11$]. Post hoc test showed that midazolam given at the dose of 0.1 μg significantly increased time spent by animals in the central sector ($P<.01$) (Table 1).

Physostigmine administered into the lateral cerebral ventricle significantly affected rat motor activity [$F(3,34)=5.62$, $P<.01$], the number of central entries [$F(3,34)=5.43$, $P<.01$], and the time spent in the central sector of the open field [$F(3,34)=5.28$, $P<.01$]. Post hoc analysis showed a significant enhancement of all exploratory parameters measured: motor activity ($P<.01$), the number of the central entries ($P<.01$), and the time spent in the central sector ($P<.05$) after 5 μg of physostigmine (Table 1).

3.1.2. Intraseptal injections

Midazolam administered into the medial septum did not show any antineophobic-like effects on rat behavior in the open field test (Table 2). Physostigmine given locally significantly increased time spent in the central sector of the open field [$F(2,24)=5.92$, $P<.01$]. The drug also showed a tendency to increase the number of central entries [$F(2,24)=2.7$, $P=.09$] and to decrease thigmotaxis [$F(2,24)=2.62$, $P=.09$]. Post hoc analysis of the data revealed that the statistically significant effect was presented after the dose of 5 μg of the drug (time in the central sector, $P<.01$) (Table 2).

3.2. Freezing reaction

3.2.1. Intracerebroventricular injections

Midazolam administered intracerebroventricularly changed, in a statistically significant way, rat spontaneously occurring freezing reactions, evaluated for 5 min immediately before contextual conditioning [$F(4,53)=3.86$, $P<.05$] (Fig. 1). The drug also significantly decreased freezing reactions examined 24 h after aversive conditioning [$F(4,53)=4.17$, $P<.05$]. The post hoc analysis of data showed that midazolam at 10 μg increased spontaneous freezing ($P<.01$), whereas the same dose decreased conditioned freezing ($P<.05$). The lower dose examined (0.5 μg icv) had no influence on unconditioned and conditioned freezing reactions ($P>.05$) (Fig. 1).

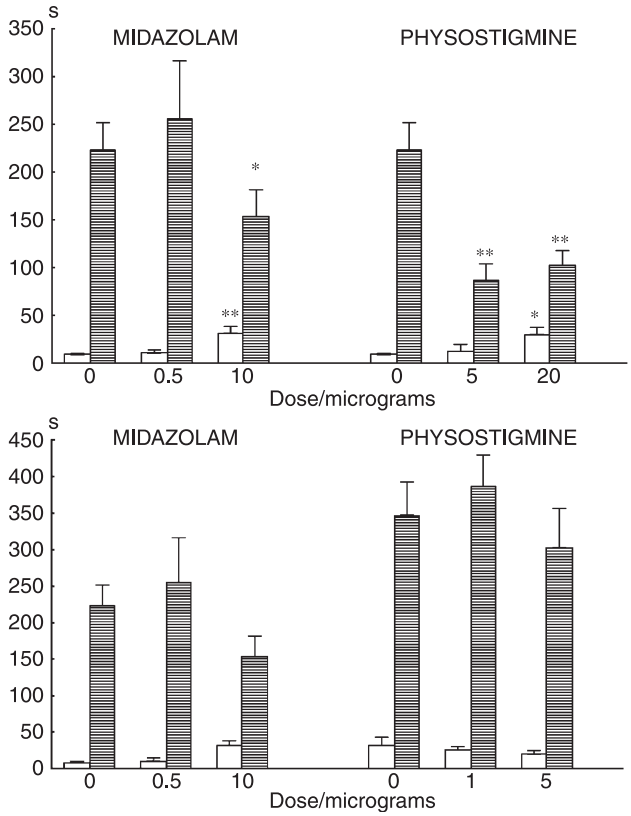

Fig. 1. The effect of intracerebroventricular (top) and intraseptal (bottom) administration of midazolam and physostigmine, on rat behavior in the contextual fear-conditioning test. The data are shown as means ± S.E.M. Ordinate: duration of freezing behavior (in seconds) recorded during preconditioning (open bars) and postconditioning (striped bars) sessions. The number of rats in each group varied from 7 to 10. *$P<.05$, differs from control group; **$P<.01$.

Physostigmine administered before a conditioning session significantly increased rat spontaneous freezing behav-

Table 2
The effect of midazolam and physostigmine administered into the medial septum (is) on rat behavior in the open field test

Drug (μg is)	*n*	Motor activity	Number of central entries	Time in the central sector (s)	Antithigmotactic effect
Midazolam					
0	15	4226.79 ± 354.00	3.00 ± 0.78	9.34 ± 2.55	65.40 ± 15.32
0.1	9	3877.22 ± 549.58	3.67 ± 1.70	9.92 ± 4.50	78.68 ± 28.58
5	9	5177.61 ± 740.01	3.44 ± 1.11	10.33 ± 3.25	82.10 ± 30.27
Physostigmine					
0	9	4653.95 ± 600.12	2.55 ± 0.77	6.86 ± 2.15	59.38 ± 15.73
1	9	5382.21 ± 420.13	2.89 ± 0.92	5.16 ± 1.37	52.40 ± 16.96
5	9	4310.10 ± 686.26	5.89 ± 1.52	30.02 ± 9.55**	115.23 ± 28.68

The data are shown as means ± S.E.M. *n* = number of rats.
** $P<.01$.

Table 3
The effect of intracerebroventricular administration of midazolam and physostigmine on rat behavior in the flinch–jump test

Drug (μg icv)	*n*	Flinch	Jump
Midazolam			
0	9	0.32 ± 0.02	0.76 ± 0.04
10.0	9	0.34 ± 0.02	0.78 ± 0.03
Physostigmine			
0	8	0.34 ± 0.04	0.61 ± 0.06
5.0	7	0.41 ± 0.08	0.59 ± 0.10
20.0	7	0.46 ± 0.06	0.73 ± 0.08

The shock threshold was measured in milliamperes. The data are shown as means ± S.E.M. *n* = number of rats.

ior [$F(4,53) = 3.86$, $P < .05$]. On the other hand, this drug significantly decreased the conditioned freezing examined 24 h after a conditioning session [$F(4,53) = 4.17$, $P < .05$]. Post hoc test revealed a significant effect of 20 μg of the drug on spontaneous freezing ($P < .05$) and of both examined doses of physostigmine on a conditioned fear reaction (5 and 20 μg icv, $P < .01$) (Fig. 1).

3.2.2. Intraseptal injections

Midazolam administered into the medial septum did not change animal freezing behavior both during a preconditioning session [$F(2,29) = 0.74$, $P = .48$] and 24 h after conditioning trial [$F(2,29) = 1.32$, $P = .33$] (Fig. 1). Intraseptal injections of physostigmine also did not change rat spontaneous freezing [$F(2,24) = 0.44$, $P = .65$] and the time of a conditioned fear reaction [$F(2,24) = 0.69$, $P = .51$] (Fig. 1).

3.3. Flinch–jump

Midazolam administered intracerebroventricularly at the behaviorally active dose of 10 μg did not change rat flinch [$t(16) = 0.65$, $df = 14$, $P = .52$] and jump [$t(16) = 0.30$, $df = 14$, $P = .77$] reactions to the painful stimuli. Similarly, physostigmine administered intracerebroventricularly did not affect the flinch [$F(2,21) = 0.83$, $P = .45$] and jump thresholds [$F(2,21) = 0.31$, $P = .74$] (Table 3).

4. Discussion

In the open field test of neophobia, both a classic benzodiazepine midazolam and an AChE-I inhibitor, physostigmine, increased at a limited dose range rat exploratory activity after intracerebroventricular injection. Physostigmine produced, in addition, the hyperlocomotory effect. Following intraseptal injections, only physostigmine selectively prolonged time spent by the animals in the central sector of the open field. In the model of a conditioned fear, both midazolam and physostigmine inhibited rat freezing reaction to the aversively conditioned context after intracerebroventricular, but not after intraseptal, pretrial drug administration. This effect was selectively related to the decrease in fear-evoked behavior, independent of changes in pain perception. In the light of this finding, and in the absence of any evidence in the literature for the direct stimulatory effect of the AChE-I on animal motor behavior (cf. Medline 1966–2001; over 5500 papers selected with physostigmine as a keyword), physostigmine enhancement of rat locomotion can be viewed as secondary to disinhibition of rat exploratory behavior. Such statement is supported by an enhancement of rat spontaneous freezing behavior after intracerebroventricular injection of similar doses of physostigmine [$F(4,53) = 3.86$, $P < .05$]. This means that physostigmine may enhance or decrease rat activity depending on a behavioral model, and that its effects are not related to the general disinhibition of animal motility.

The presented data confirmed the selective influence of physostigmine on the central emotional processes. In the earlier paper, the anxiolytic-like potency of physostigmine was found to be at least as strong as that of the benzodiazepine derivative midazolam (Sienkiewicz-Jarosz et al., 2000). It was also previously reported that microinfusion of midazolam into the lateral but not the medial septum suppressed fear reactions in two tests of rat anxiety, and this effect was partially blocked by the benzodiazepine receptor antagonist Ro 15-1788 (Pesold and Treit, 1996). These results indicate that the anxiolytic-like effects of intraseptal midazolam occur, at least in part, at $GABA_A$/benzodiazepine receptor sites located in the lateral septal nuclei. Lateral septum has also been implicated in the action of nicotine and 8-OH DPAT (a 5-HT_{1A} receptor agonist), modulating rat behavior in the social interaction and elevated plus maze tests of anxiety (Cheeta et al., 2000a,b). The above mentioned data concur with the immunohistochemical studies of the c-Fos gene expression, used to map functional activation in discrete brain regions of rats processed in different models of anxiety (Campeau et al., 1997; Duncan et al., 1996). The results indicate that specific forebrain regions were affected, including the medial prefrontal cortex, medial amygdala, and lateral septum. However, microinjections of the $GABA_A$ receptor agonist muscimol into either the lateral or the medial septum increased rats' open-arm exploration in the plus maze and decreased their burying behavior in the shock-probe test (Degroot et al., 2001). It appeared, therefore, that both septal areas, lateral and medial, act in concert in the control of anxiety, but they differ in terms of intrinsic mechanisms involved in modulation of different types of emotional reactions. Accordingly, microinjections of neuropeptide Y or the neuroactive steroid pregnanolone into the lateral septum produced selective anxiolytic effects in some animal models of anxiety only (Bitran et al., 1999; Kask et al., 2001). The observed lack of the effects of intramedial septum administration of midazolam in the present study supports the other authors' findings about a selective involvement of $GABA_A$/benzodiazepine receptors in the lateral septum nuclei in processing of emotional input (Pesold and Treit, 1996).

The presented results clearly indicate that the cholinergic system in the medial septum significantly contributes to emotional processes controlling rat behavior in the test of unconditioned neophobia, but not in the model of a conditioned fear reaction. Similarly, physostigmine was reported to increase unconditioned open-arm exploration in the plus maze test and decrease burying behavior in the shock-probe test after direct intrahippocampal injections (Degroot et al., 2001). Altogether, these findings point to the hippocampal cholinergic system, mostly represented by the septo-hippocampal cholinergic pathway (Aloisi, 1997; Nicoll, 1985; Wainer et al., 1985), as playing a modulatory role in emotions. The effect of physostigmine was selective, independent of changes in pain perception and learning-related processes (such conclusion can be inferred from the results of the pretrial administration of this procognitive drug in the conditioned fear test).

To summarize, the presented data add more arguments from the basic research point of view for the selective anxiolytic-like action of AChE-Is, represented by a prototypical drug physostigmine, in the models of neophobia and contextual fear. It seems, therefore, that the calming and sedative effects of AChE-Is, observed in patients with Alzheimer's disease, may be directly related to their anxiolytic action, independently of an improvement in cognition that may decrease novelty-induced, disorientation-related distress and anxiety.

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