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Behavioral side effects in rats treated with acetylcholinesterase inhibitors suggested used as prophylactics against nerve agents

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ABSTRACT

Acetylcholinesterase inhibitors in combination with an anticholinergic, particularly anticholinergics with antiglutamatergic properties, can effectively protect against nerve agent-induced seizures and lethality. The objective of the present study was to examine potential behavioral side effects of the anticholinesterases physostigmine (0.1 mg/kg), galantamine (3 mg/kg), huperzine (0.5 mg/kg), and donepezil (2.5 mg/kg) alone or each drug in combination with anticholinergic procyclidine (3 mg/kg). The results showed that rats injected intraperitoneally with galantamine displayed a mild cognitive deficit in terms of reduced preference for novelty that was similarly found among animals treated with procyclidine combined with either galantamine or donepezil. Locomotor activity and rearing were radically depressed in all groups treated with anticholinesterases as well as in combination with procyclidine. Reductions in activity were most prominent for rats injected with galantamine alone. Equalizing effects of cholinesterase inhibitors and anticholinergics were absent in the present context. Findings from previous studies that both systemic and local (amygdala) application of physostigmine cause increased fear-motivated freezing response in rats, may explain the marked reductions in activity among the present rats. In view of these findings, use of anticholinesterases (crossing the blood-brain barrier) as prophylactics against nerve agents must be carefully examined to avoid severe side effects.

1. Introduction

Organophosphorus nerve agents are lethal chemical warfare means, that may be encountered during military combats, terrorist use, or during chemical disarmament. Nerve agents act by irreversibly inhibiting acetylcholinesterase, the enzyme that hydrolyzes acetylcholine. Accumulation of acetylcholine results in excessive stimulation of muscarinic and nicotinic receptors. The signs of poisoning are seen as increased salivation, respiratory distress, tremor, seizures/convulsions, coma, and death. Increased cholinergic activity in the brain is probably related to the initial phase of seizures (McDonough and Shih, 1997; Lallement et al., 1992), whereas sustained seizures are probably associated with increased glutamatergic activity leading to neuronal damage predominantly in the hippocampus, amygdala, piriform cortex, and entorhinal cortex (McDonough and Shih, 1997; Carpentier et al., 1991).

In order to prevent lethality by soman [-(1,2,2-trimethyl-propyl) methyl-phosphonofluoridate] it is important to shield temporarily a portion of the acetylcholinesterase from irreversible inhibition followed by the therapeutic treatment with an anticholinergic drug. To meet these requirements, a number of military forces have based their medical therapy on pyridostigmine pretreatment to prevent acetylcholine inhibition by nerve agents followed by the immediate therapeutic treatment with atropine sulfate and an oxime administered by one or more autoinjectors. These drugs are intended to inhibit muscarinic receptors and to reactivate any “unaged” enzyme, respectively, following exposure to nerve agent (Aas, 2003). However, since pyridostigmine does not readily cross the blood-brain barrier, physostigmine that readily enters the brain, has been suggested as a possible replacement. In studies of guinea pigs and rats, evidence has been presented that effective prevention of soman-induced lethality can be assured by physostigmine in combination with scopolamine or procyclidine (Kim et al., 2002; Choi et al., 2004; Myhrer et al., 2004b, Philippens et al., 2000; Wetherell et al., 2002). Pyridostigmine combined with caramiphen or benactyzine and trihexyphenidyl or with biperiden have also been reported to provide efficacious pretreatment in soman-poisoned rats (Bajgar, 2004; Kassa et al., 2003; Raveh et al., 2003).

The half-life of physostigmine is relatively short. For this reason, the Alzheimer drugs donepezil, galantamine, and huperzine with relatively long half-lives have been suggested as possible alternative prophylactic cholinesterase inhibitors against nerve agent intoxication (Aas, 2003). Donepezil is a partial reversible centrally acting and highly selective inhibitor of the acetylcholinesterase (Sugimoto et al., 2002). Galantamine is another drug approved for treatment of mild to moderate Alzheimer's disease. The drug is a reversible acetylcholinesterase inhibitor that crosses the blood-brain barrier (Corey-Bloom, 2003).

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Huperzine is a slow, reversible inhibitor of the acetylcholinesterase at both peripheral and central levels (Ashani et al., 1992). This drug is used for treatment of Alzheimer's disease in China (Wang et al., 2000).

Acetylcholinesterase inhibitors are not very efficacious prophylactics against nerve agent poisoning if they are administered alone. Their efficacy is considerably enhanced when combined with an anticholinergic agent like atropine. However, the anticonvulsant impact may be even further improved if acetylcholinesterase inhibitors are administered along with an antiparkinson drug. This group of agents possesses potent anticonvulsant properties against nerve agents, because the drugs exert both cholinergic and glutamatergic antagonism in mice and rats (Gao et al., 1998; McDonough and Shih, 1995; Raveh et al., 2002). The antiglutamatergic effect appears particularly relevant, since glutamatergic pathways have been suggested to be intimately involved in the early stages of soman-induced seizures (Weissman and Raveh, 2008). For the present purpose, procyclidine was chosen. This drug combined with either physostigmine or donepezil can effectively prevent soman-generated seizures and lethality in rats (Kim et al., 2002; Haug et al., 2007; Myhrer et al., 2004b). Procyclidine does not seem to have been examined in combination with galantamine or huperzine in previous nerve agent studies.

Because seizures are associated with both lethality and brain damage (Shih et al., 2003), it is very important to prevent the onset of seizures or terminate seizures within 20 min after onset to avoid neuropathology (Lallement et al., 1994; McDonough et al., 1995). However, a crucial matter is whether the doses of prophylactics required for protection of military personnel against nerve agent-induced damage will impair cognitive functions. The purpose of the present study was to make a comparative assessment of potential behavioral effects of procyclidine, donepezil, galantamine, huperzine, and physostigmine (Experiment 1) or each acetylcholinesterase inhibitor in combination with procyclidine (Experiment 2). The doses of drugs chosen have previously been shown to have anticonvulsant effects against soman-induced seizures. The behavioral task employed was a novelty test that has proven particularly sensitive in revealing cognitive dysfunctions following selective disruptions of entorhinal projections (Myhrer, 1988, 1989). Exploration of a discrete novel object is one form of inquisitive activity frequently seen among rats. This activity appears as a strong preference for novelty, the recognition of which is probably based on polymodal sensory information (Berlyne, 1960). The rats were tested in a modified version of the novelty test of Berlyne (1950) consisting of three different sets of stimuli; visual/tactile, olfactory, or visual only (Myhrer, 1988).

2. Materials and methods

2.1. Animals

2.1.1. Experiment 1

Forty-eight male Wistar albino rats from a commercial supplier (Taconic Breeding Laboratories, Denmark) weighing 280–310 g when the experiment started, served as subjects. The rats were randomly assigned to one of 6 groups (8 rats in each) and their group assignment was unknown during testing. The various groups received i.p. injection of either saline, procyclidine, donepezil, galantamine, huperzine, or physostigmine. The rats were housed individually and had free access to commercial rat pellets and water. With the novelty test used, reliable results are dependent on emotionally stable animals. For this reason, the rats were handled individually 7–10 days, being allowed to explore a table top (80×60 cm) for 3 min a day. The climatized (21 °C) vivarium was illuminated from 0700 to 1900 h.

2.1.2. Experiment 2

Forty male Wistar rats (280–310 g) from the same supplier served as subjects. The animals were randomly assigned to one of 5 groups with 8 rats in each. The various groups received i.p. injection of saline or procyclidine combined with either donepezil, galantamine, huperzine, or physostigmine. The rats were treated as described for Experiment 1.

The experiments were approved by the National Animal Research Authority. A minimal number of animals were used, and all efforts were made to avoid animal suffering according to the European Communities Council Directive of 1986 (86/609/EEC).

2.2. Drug administration

Physostigmine salicylate (Sigma-Aldrich) was dissolved in physiological saline (0.9%) and administered in standardized dose of 0.1 mg/kg (Myhrer et al., 2004b). Donepezil hydrochloride was obtained as 5 mg tablets (Aricept®, Pfizer). The tablets were crushed, suspended in saline (2 mg/ml) and given in a dose of 2.5 mg/kg (Haug et al., 2007). Although donepezil is water soluble, the suspension was injected instead of just the aqueous extract to ensure a homogenous administration. Huperzine A (Sigma-Aldrich) was dissolved in saline and injected in a dose of 0.5 mg/kg (Tonduli et al., 2001). Galantamine hydrobromide (Sigma-Aldrich) was dissolved in saline and given in a dose of 3 mg/kg that attenuates cognitive impairment induced by medial septal lesions in rats (Mulder et al., 2005). Galantamine does not seem to have been used against soman in rats. In guinea pigs, however, anticonvulsant doses of 5 or 8 mg/kg of galantamine have been used against soman (Albuquerque et al., 2006). Thus, the dose of 3 mg/kg for rats appears rather conservative. Procyclidine hydrochloride (Sigma-Aldich) was dissolved in saline and administered in a dose of 3 mg/kg (Myhrer et al., 2004a). The drugs were given 20 min before each test session (one session a day for 3 days) with a total testing period of 20 min. One exception was huperzine that was given 40 min before each test session, because stable acetylcholinesterase inhibition is obtained after 40 min in rats (Tonduli et al., 2001). When procyclidine was combined with anticholinesterases (Experiment 2), the injections were given in rapid succession (procyclidine first, also in combination with huperzine). Physiological saline was injected i.p. in a volume of 0.3 ml. Prophylactics are usually given 20 or 30 min before exposure to nerve agent (Myhrer et al., 2008).

2.3. Apparatus

Behavioral testing was carried out in a Plexiglas cage (54×33×20 cm) previously described (Myhrer, 1988). In brief, the floor was divided in 18 equal squares (9×11 cm). Three identical aluminum cubes (5×5×5 cm) were evenly distributed in the cage in fixed positions (the neutral objects). Three other cubes made up the novel objects. One object only differed from the neutral ones in that its top was uneven with tracks (2 mm) in it making up a square pattern (visual/tactile stimuli). Since the rats could perceive the tracks or the squares (16 squares measuring 1.1×1.1 cm) by bodily contact, both tactile and visual sensory modalities might be used. One was identical with the neutral ones, and a spot of cheese (dia. 1.5 cm) was smeared on the side facing the experimenter (olfactory stimulus). So-called Norwegian white cheese (Norvegia) that hardly smells at all to humans was used. In the test cage, it was not possible to detect the cheese visually. One was smaller than the neutrals, (4.5×4.5×4.5 cm) and two sides were slightly uneven (visual stimulus). All objects were painted light gray. The sound attenuated testing room was provided with a fan producing white noise (52 dB).

2.4. Procedure

The same procedure was followed for both Experiments 1 and 2. During adaptation, the rats were allowed to explore individually the empty apparatus for 20 min. On the next day, the rats were given the test drugs before they were run in Session I. In Phase 1, the animals were tested for 5 min in the test cage with three neutral objects present. Then the rats spent 10 min in the home cage. In Phase 2, the rats were tested again for 5 min, and the neutral object in the middle

Table 1
Mean (±SEM) measures of exploratory behavior in seconds in novelty test in Experiment 1.

			Differential time exploring[a]			Total time exploring					
			Session			Session					
			I	II	III	I		II		III	
Group	*N*	Dose (mg/kg)	Ph 2	Ph 2	Ph 2	Ph 1	Ph 2	Ph 1	Ph 2	Ph 1	Ph 2
Saline	8	–	5.3±3.0	20.6±3.4	16.0±2.2	17.8±2.6	15.6±3.0	14.0±2.1	30.3±6.7	14.1±1.9	22.1±2.0
Procyclidine	8	3.0	3.6±1.6	13.9±2.7	12.0±2.3	16.8±3.7	10.4±2.5	13.5±2.0	20.6±3.2	14.5±2.4	20.3±2.4
Donepezil	8	2.5	5.5±2.5	16.2±3.3	13.7±3.7	19.6±4.2	17.9±4.9	11.8±3.3	22.8±3.5	16.0±2.6	22.9±4.6
Galantamine	8	3.0	2.8±1.1	7.3±1.6*	7.0±2.3	14.0±1.9	5.3±1.8	12.6±3.0	15.3±2.1	11.6±1.6	12.1±2.3
Huperzine	8	0.5	6.7±4.2	24.3±3.2	13.3±1.8	15.1±4.2	15.1±4.3	15.9±3.5	31.3±3.7	16.9±2.8	20.3±1.7
Physostigmine	8	0.1	4.4±1.5	17.4±3.6	16.3±3.0	18.8±2.3	7.4±2.2	16.3±3.6	21.1±4.2	18.1±4.9	25.0±3.8

Ph = phase. Significantly different from the saline group: *$P<0.05$.
[a] Difference in time between exploring novel and neutral items.

position had been replaced by the novel object with uneven top. Changing position of neutral object makes up a novelty in itself (Ennaceur et al., 1996). Preference for novelty was based on the difference between exploration of novel versus neutral objects, and the mean time of contact with the two neutral objects was used. During Phases 1 and 2 the following behaviors were recorded: number of seconds in contact with the objects, number of squares traversed (locomotor activity), and number of rearings. Exploration of an object was defined as directing the snout toward the object at a distance of 1.5 cm or less. Bodily touch other than by the snout was not considered as exploratory behavior. Prior to testing of each rat the apparatus and objects were carefully washed with Zalo (Lilleborg, Norway) dissolved in water and allowed to dry. In Sessions II and III (test days 2 and 3), the same procedure was followed, and the novelty was represented by smell of cheese on one side of the cube and a smaller object, respectively. Since changing the order of novelty presentation can lead to different patterns of locomotor and rearing activity, a counterbalanced order of testing was not used to control for accumulative effects of drugs on activity measures. The same set of neutral cubes was used after olfactory cues had properly been eliminated. One observer, who was unaware of the rats' group assignment, recorded the data manually without TV monitoring.

2.5. Statistics

Overall analyses were carried out with one-way or two-way analysis of variance (ANOVA). Group comparisons were made with Newman–Keuls post hoc test. Computations were made with Prism statistical software program (GraphPad Software CA, USA).

3. Results

3.1. Experiment 1

Decreased preference for novelty was seen among the rats treated with galantamine (Table 1). In Session II (smell novelty), one-way ANOVA showed a reliable overall effect ($F(5,42)=3.722$, $P=0.007$). The galantamine group displayed a significant preference deficit relative to the control group ($P<0.05$) as well as the huperzine group ($P<0.01$). The total time exploring objects did not differ significantly among the groups (Table 1).

As seen from Fig. 1A, rats treated with acetylcholinesterase inhibitors tended to display less motor activity than the control animals and animals treated with procyclidine. Two-way ANOVA revealed a significant Group × Time (Session/Phase) interaction ($F(5,25)=9.41$, $P=0.0076$), a significant between group factor ($F(5,25)=14.16$, $P<0.0001$), as well as a significant within group factor ($F(5,25)=26.43$, $P<0.0001$). The significance levels in Fig. 1 are relative to the performances of the control group and are based on one-way ANOVA followed by group comparisons with Newman–Keuls post hoc test. Beyond the results in Figs. 1 and 2, significant differences between the groups treated with drugs are presented in this section. In Phase 1 in Session I, the galantamine, huperzine, physostigmine, and donepezil groups were reliably less active than the procyclidine group ($P<0.05$). The galantamine group was significantly less active than the donepezil and physostigmine groups ($P<0.05$). In Phase 2 in Session I, the galantamine and physostigmine groups were less active than the procyclidine group ($P<0.01$). In Phase 1 in Session II, the galantamine group exhibited less locomotor activity than the huperzine group ($P<0.05$). In Phase 1 in Session III, the galantamine and physostigmine groups were less active than the huperzine and procyclidine groups ($P<0.05$).

The rearing activity also differed among the groups (Fig. 1B). Two-way ANOVA revealed a significant Group × Time (Session/Phase)

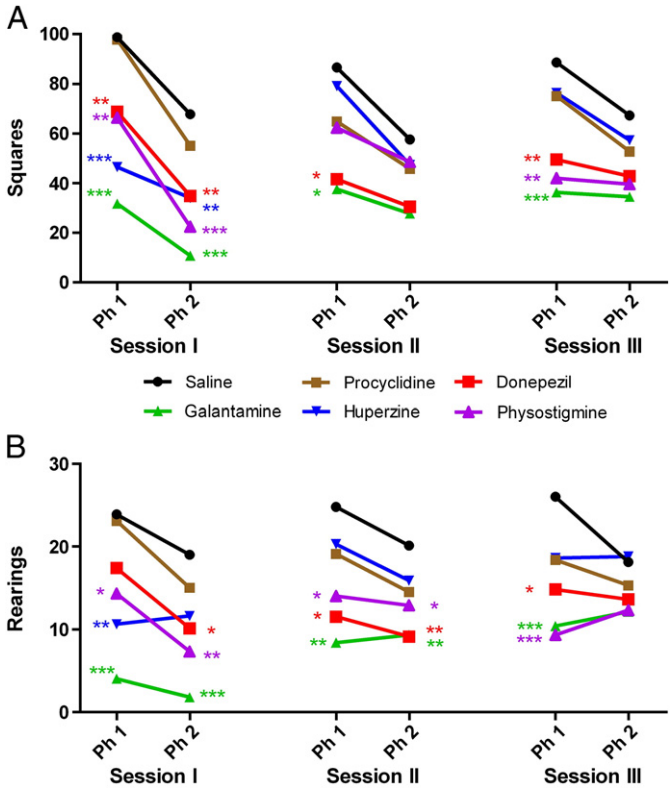

Fig. 1. Mean measures of locomotor activity (A) and rearing activity (B) in Experiment 1. Significance levels are based on ANOVA followed by group comparisons with Newman–Keuls post hoc test and show differences relative to the saline group. Ph = phase. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

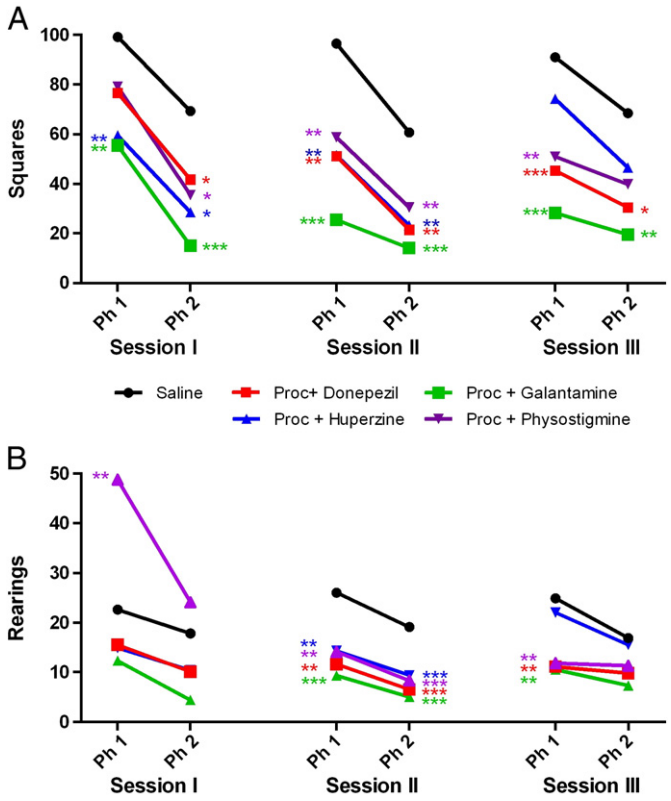

Fig. 2. Mean measures of locomotor activity (A) and rearing activity (B) in Experiment 2. Significance levels are based on ANOVA followed by group comparisons with Newman–Keuls post hoc test and show differences relative to the saline group. Ph = phase, Proc = procyclidine. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

interaction (F (5,25) = 9.05, $P = 0.0293$), a significant between group factor (F (5,25) = 5.40, $P = 0.0003$), as well as a significant within group factor (F (5,25) = 30.37, $P<0.0001$). In Phase 1 in Session I, the galantamine group made significantly less rearing than the procyclidine group ($P<0.001$), donepezil group ($P<0.01$), and physostigmine group ($P<0.05$). In Phase 2 in Session I, the galantamine group made reliably less rearing than the procyclidine, huperzine, and donepezil groups ($P<0.05$). In Phase 1 in Session II, the galantamine group made less rearing than the huperzine and procyclidine groups ($P<0.05$).

3.2. Experiment 2

Decreased preference for novelty was seen in several groups (Table 2). In Session II, ANOVA disclosed a reliable treatment effect (F (4,35) = 3.452, $P = 0.017$). The procyclidine + donepezil group displayed significantly less preference for novelty than the control group ($P<0.01$). In Session III (smaller object novelty), ANOVA showed a significant difference among the groups (F (4,35) = 3.340, $P = 0.024$). The procyclidine + galantamine group explored the novel object reliably less than the control group and the procyclidine + donepezil group ($P<0.05$).

The total time exploring objects differed among the groups (Table 2). ANOVA showed a significant treatment effect in Phase 2 in Session I (F (4,35) = 4.470, $P = 0.0051$). The procyclidine + donepezil group explored objects reliably less than the saline group ($P<0.05$) and the procyclidine + huperzine group ($P<0.01$). The procyclidine + galantamine group also explored objects less than the procyclidine + huperzine group ($P<0.05$). In Phase 1 in Session II, a reliable treatment effect occurred among the groups (F (4,35) = 4.832, $P = 0.0033$). The procyclidine + galantamine group explored objects significantly less than the procyclidine + physostigmine group ($P<0.01$) and the procyclidine + huperzine group ($P<0.05$). The procyclidine + donepezil group also explored significantly less than the procyclidine + physostigmine and procyclidine + huperzine groups ($P<0.05$). In Phase 2 in Session II, ANOVA disclosed a reliable overall effect (F (4,35) = 4.048, $P = 0.0048$). Relative to the control group the procyclidine + donepezil group made reliably less exploring ($P<0.01$) as was also seen for the procyclidine + galantamine group ($P<0.05$). In Phase 1 in Session III, a significant treatment effect was observed (F (4,35) = 6.434, $P = 0.0005$). The procyclidine + physostigmine group explored objects reliably more than all the other groups ($P<0.05$). In Phase 2 in Session III, ANOVA revealed a significant overall effect (F (4,35) = 4.058, $P = 0.0083$). Compared with the control group the procyclidine + galantamine group made reliably less exploring of objects ($P<0.01$) as also seen for the procyclidine + huperzine group ($P<0.05$).

The rats treated with combination of drugs tended to display reduced locomotor activity (Fig. 2A). Two-way ANOVA revealed a significant Group × Time (Session/Phase) interaction (F (4,20) = 23.81, $P<0.0001$), a significant between group factor (F (4,20) = 11.52, $P<0.0001$), as well as a significant within group factor (F (5,20) = 15.81, $P<0.0001$). In Phase 1 in Session II, the procyclidine + galantamine group showed significantly less locomotor activity than the procyclidine + physostigmine group ($P<0.05$). In Phase 1 in Session III, the procyclidine + huperzine group was more active than the procyclidine + galantamine group ($P<0.001$) and the procyclidine + donepezil, physostigmine groups ($P<0.05$).

Fig. 2B shows the rearing activity among the groups. Two-way ANOVA revealed a significant Group × Time (Session/Phase) interaction (F (4,20) = 19.18, $P<0.0001$), a significant between group factor (F (4,20) = 10.59, $P<0.0001$), as well as a significant within group factor (F (5,20) = 15.83, $P<0.0001$). In Phase 2 in Session I, the procyclidine + galantamine group made significantly less rearing than the procyclidine + physostigmine group ($P<0.01$). In Phase 1 in Session III, the procyclidine + galantamine, donepezil, or physostigmine groups made less rearing than the procyclidine + huperzine group ($P<0.05$).

Table 2
Mean (±SEM) measures of exploratory behavior in seconds in novelty test in Experiment 2.

		Differential time exploring[a]			Total time exploring					
		Session			Session					
		I	II	III	I		II		III	
Group	*N*	Ph 2	Ph 2	Ph 2	Ph 1	Ph 2	Ph 1	Ph 2	Ph 1	Ph 2
Saline	8	7.3 ± 3.3	24.8 ± 7.2	12.2 ± 2.4	19.0 ± 2.6	14.4 ± 3.5	11.5 ± 1.9	32.8 ± 7.6	12.9 ± 2.3	22.1 ± 2.0
Proc + donepezil	8	0.1 ± 1.0	3.0 ± 1.1**	11.4 ± 3.6	13.4 ± 2.0	4.0 ± 1.1*	6.6 ± 1.7	5.4 ± 1.3**	9.0 ± 2.6	15.8 ± 3.1
Proc + galantam	8	2.3 ± 1.2	10.4 ± 5.3	1.9 ± 1.1*	15.6 ± 2.9	7.5 ± 1.7	5.5 ± 2.1	12.1 ± 5.4*	5.8 ± 1.3	7.0 ± 2.0**
Proc + huperzine	8	3.5 ± 2.1	13.1 ± 2.7	5.4 ± 2.2	14.0 ± 4.0	17.8 ± 3.9	16.4 ± 3.3	16.6 ± 3.5	11.0 ± 2.4	11.3 ± 3.0*
Proc + physostig	8	0.0 ± 1.9	9.0 ± 2.1	10.0 ± 1.9	20.8 ± 2.9	12.0 ± 1.5	18.9 ± 3.8	16.9 ± 4.9	21.4 ± 2.7*	16.8 ± 3.7

Galantam = galantamine, Ph = phase, physostig = physostigmine, proc = procyclidine. Significantly different from the saline group: *$P<0.05$, **$P<0.01$.
[a] Difference in time between exploring novel and neutral items.

4. Discussion

The results from the present study showed that cognitive impairment in terms of reduced preference for novelty was seen in the galantamine group during one session only in Experiment 1 and when procyclidine was combined with either galantamine or donepezil in Experiment 2. The total time of exploring objects was unaffected by all acetylcholinesterase inhibitors, whereas the total time exploring was occasionally changed in all groups with combined treatments. The locomotor and rearing activities were markedly reduced in all groups treated with anticholinesterases alone as well as in the combination with procyclidine. The latter finding appears somewhat intriguing in view of the expectation that effects of cholinesterase inhibitors and anticholinergics may offset each other (Kim et al., 2002).

Increased cholinergic activity produced by cholinesterase inhibitors is supposed to enhance cognitive performance. In correspondence with this view, physostigmine improves the performance on the radial maze in normal rats (Ennaceur, 1998). It has also been shown that anticholinesterases can compensate for impaired cognition. Both donepezil, galantamine, and huperzine can attenuate experimentally-induced cognitive deficits in various tasks in rats (Higgins et al., 2002; Mulder et al., 2005; Wang et al., 2000). The reduced preference for smell of cheese among the galantamine rats can hardly be attributed to decreased olfactory perception, because preference for smell was evident when galantamine was combined with procyclidine. The deficits in preference for novelty seen in the groups with combined treatments of procyclidine and donepezil or procyclidine and galantamine may be related to the occurrence of low level of total time exploring objects for these groups. This relationship may reflect an attenuated interest in items in general.

When considering all behavioral measures collectively, physostigmine and huperzine produced the least detrimental effects, and galantamine produced the most detrimental effects. A tremendous depression of locomotor activity and rearing was found for all groups treated with anticholinesterases relative to the saline and procyclidine groups (Fig. 1). Even if the reductions in activity were rather uniform for all groups, galantamine depressed locomotion and rearing even more thoroughly than other cholinesterase inhibitors. When the anticholinesterases were combined with procyclidine, the activity reducing effects were even more pronounced (Fig. 2). One exception was the increased rearing seen among rats treated with procyclidine and physostigmine in Phase 1 of Session I; a result not readily accounted for. Also in the combination with procyclidine galantamine more powerfully than other combinations reduced locomotor and rearing activities. These findings suggest that the cholinergic antagonism of procyclidine did not counteract the agonistic effect of cholinesterase inhibitors for functions involved in locomotion and rearing. It might be argued that the procyclidine dose of 3 mg/kg was too low for effects of counteraction to become evident. However, a high dose of procyclidine (6 mg/kg) combined with physostigmine (0.1 mg/kg) results in marked cognitive impairment along with suppressed locomotor and rearing activities even more pronounced than for 3 mg/kg of procyclidine in the present test situation (Myhrer et al., 2004a). We have previously shown that some antiparkinson drugs like benactyzine, caramiphen, and trihexyphenidyl can exert marked cognitive deficits in the present novelty test, but when physostigmine is coadministered with each of the antiparkinson agents referred to above, the cognitive impairment disappears (Myhrer et al., 2008). There are, however, exceptions to this counteracting principle, because a very potent anticholinergic (scopolamine) or a high dose of procyclidine (6 mg/kg) still results in cognitive deficits in spite of coadministration with physostigmine (Myhrer et al., 2004a). From this comparison of previous and present results, it appears that cognitive and psychomotor processes can be affected in different ways by the combination of an anticholinergic and a cholinesterase inhibitor.

A simple antagonism between an anticholinergic and anticholinesterases did not occur in the present study. Since the half-life of drugs may vary, the subtle balance required for such equalizing effect would be hard to achieve for nerve agent pretreatment. One way to circumvent the problem might be continuous delivery of drugs by means of minipump or dermal patch. In the present study, not only a lack of drug antagonism was seen, but even increased decline in activity and rearing resulted from the combination of procyclidine and anticholinesterases (Figs. 1 and 2).

The doses of acetylcholinesterase inhibitors used in the present study were selected because they have previously been shown to cause anticonvulsant efficacy against soman intoxication (cf., Drug administration). Whether these doses produced equivalent levels of acetylcholinesterase inhibition in red blood cells was not examined. Hence, the finding that galantamine led to more severe behavioral changes than the other anticholinesterases may be related to a dose effect.

Locomotor activity and rearing express exploratory behavior in rodents. The innate curiosity of rats makes them avoid stimulus reexposure by continuously exploring new places and items (Berlyne, 1960). The dramatic decline in locomotor and rearing activities produced by cholinesterase inhibitors alone or in combination with procyclidine may be associable with several potential causes. A general motor deficit is hardly attributable to the reduced activity levels, because intact locomotion and rearing were seen during several phases in all groups treated with anticholinesterases (Fig. 1). For the same reason, reduced locomotor and rearing activities cannot be ascribed deficits in sensory processing. Remaining potential explanations may be decreased curiosity or increased fear. However, almost intact preference for novelty and unaffected total time exploring objects among the groups treated with anticholinesterases probably rule out decreased curiosity as an explanation. Retained curiosity may explain the apparent paradox of unaffected total time exploring concurrent with reduced locomotor activity and rearing.

Rats appear to have innate defensive reactions, such as freezing, flight and threat, which they display in response to predators and aversive stimuli (Bolles, 1970). Results from experiments with scopolamine suggest that cholinergic synapses may be involved in the mediation of these defensive responses (Plotnik et al., 1974). In a subsequent study, intraperitoneal administration of various doses of physostigmine (0.025–0.2 mg/kg) resulted in a dose-related increase of freezing, suppression of feeding, and suppression of time near aversive stimulus (Mollenauer et al., 1979). In the latter study, it was suggested that the effect of physostigmine is not to depress behavior in general, but rather to increase or potentiate the innate defensive response of freezing. Thus, the freezing effect is central to other changes. More recent research has focused on cholinergic activity in the emotion regulating amygdaloid complex. Microinfusion of physostigmine into the basolateral amygdala increases the time spent freezing in intact rats. This finding indicates that muscarinic activation of amydaloid input from the nucleus basalis magnocellularis influences fear-motivated freezing behavior (Power and McGaugh, 2002). In correspondence with the latter view, reduced freezing induced systemically by scopolamine can be reinstated by donepezil (Lindner et al., 2006). A plausible explanation of the present findings of remarkably decline in locomotor and rearing activities may be that the anticholinesterases activated the freezing response. This interpretation receives support from the present data, inasmuch as both locomotion and rearing were normal during the last phase in Session III (Fig. 1) when the adaptation to the test situation was optimal. Natural fear in the present rats had been markedly reduced by thorough handling and by using a type of test apparatus that previously had served as colony cage.

It has been well documented that the anticholinesterases used in this study can mitigate symptoms of Alzheimer's disease (cf., Introduction). However, cholinesterase inhibitors used in healthy persons can have perturbing effects. The influence of physostigmine on stimulus-selectivity and/or task-related responses is often opposite between Alzheimer patients and healthy controls. In control subjects, excessive

cortical activation (functional magnetic resonance imaging-scanning) during task-irrelevant conditions occurs in addition to enhanced cholinergic activation in the frontoparietal and sensory cortex during low-attention conditions that do not normally engage such brain areas (Bentley et al., 2008). These results support a model of anxiety in which increased release of cortical acetylcholine augments the expression of fear and anxiety (Berntson et al., 1998). In the latter model, neuronal links between the basal forebrain cortical cholinergic system, basolateral amygdala, and cardiovascular reactivity make up fundamental elements. It has been demonstrated that anxiety is associated with exposure to organophosphate compounds. Commercial pesticide sprayers show elevated anxiety and lower plasma cholinesterase than control subjects (Levin et al., 1976). Hence, there is an apparent correspondence between the findings of increased fear/freezing in animals and elevated anxiety in humans following exposure to cholinesterase inhibitors. Neurocognitive deficits, neuroendocrine alterations as well as anxiety and mood alterations in Gulf War veterans have been attributed to the use of pyridostigmine and pesticides during deployment (Research Advisory Committee on Gulf War Veterans' Illnesses, 2008).

In conclusion, marked suppression of locomotor activity and rearing may be generated by fear-motivated freezing in response to the administration of centrally active anticholinesterases alone or in combination with procyclidine. The behavioral inhibition obtained suggests that cholinesterase inhibitors may not be suitable as prophylactics against nerve agents.

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