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Research report

Repeated stress in combination with pyridostigmine Part I: Long-term behavioural consequences

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ABSTRACT

Since their return from the first Persian Gulf War, some veterans have complained of a variety of symptoms that were designated as "Gulf War Illness" (GWI). Among other factors, pyridostigmine, used as a prophylaxis treatment against intoxication by nerve agents, has been proposed by many authors as a cause of late social and/or cognitive dysfunction related to GWI. One of the hypotheses placed to explain these behavioural disorders is that operational stress has modified the side effects of pyridostigmine given to soldiers. In an attempt to establish an experimental model of GWI to evaluate the long-term behavioural effects of pyridostigmine administered in stressful conditions, we have developed a new model of repeated stress based on the pole-climbing avoidance technique. We used it to evaluate the effects of pyridostigmine treatment combined to repeated stress over the months following the end of the treatment. We observed that this stress induces impulsiveness and aggressiveness in adult male rat. Moreover, pyridostigmine treatment administered daily 30 min before each stressful session amplifies these behavioural disorders and induces long-term learning dysfunction and slight but significant decrease in phosphocholine level in hippocampus. This suggests that repeated administration of pyridostigmine combined to pole-climbing avoidance (PCA) stress conditions can induce adverse effects in rat central nervous system.

1. Introduction

Since their return from the first Persian Gulf War (PGW), some veterans have complained of a variety of symptoms that could not be associated with a single disease and hence were designated as "Gulf War Illness" (GWI) (for review see [12]). These symptoms include chronic fatigue, musculo-skeletal pain and cognitive-psychological disturbances such as memory loss, confusion, inability to concentrate, mood swings, particularly irritability and somnolence [76,31,20,61,39,18,86,14,49]. Among other factors (for review see [27]), pyridostigmine bromide (PB), used as a prophylaxis treatment against intoxication by nerve agents, has been proposed by many authors as a cause of late social and/or cognitive dysfunction related to GWI [68,28–31,22]. One of the hypotheses placed to explain these behavioural disorders is that operational stress has modified the effects of PB prophylactically given to soldiers in order to prevent potential chemical intoxication. This hypothesis is supported by epidemiological [26,83,75] and experimental studies showing that PB could amplify the early effects of acute or sustained stress in rodents [19,38,5,77]. These findings strengthen the idea that during the PGW, PB in combination with other factors such as stress may have played a role in the appearance of side effects and may have even influenced pretreatment efficacy. This emphasizes the need for considering the efficacy and side effects of a drug under stressful conditions. This has been demonstrated for the combination of physostigmine (PHY, another acetylcholinesterase inhibitor) and scopolamine (SCO) as a pretreatment against soman intoxication in chronically stressed guinea pigs [59,60]. Similarly Shaikh and Pope reported that daily forced running increased signs of peripheral cholinergic toxicity following subacute PB treatment [67].

To our knowledge, no studies were conducted to describe the effects of repeated stress combined with repeated PB administration on behaviour. In an attempt to establish an experimental model of GWI and evaluate the long-term behavioural effects of pyridostigmine administered in stressful conditions, we have developed a new experimental model of repeated stress based on the pole-climbing avoidance technique [43]. Then we combined it with PB treatment to evaluate the side effects of PB administered in

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Table 1
Study design

Day (D)	Test, treatment, samplings		
	Experiment A ($n = 80$)	Experiment B ($n = 80$)	Experiment C ($n = 32$)
D1 to D5	PB treatment (1.5 mg/kg/day, 5 ml/kg, *p.o.*) then PCA test (30 min later)		
D6 and D7	REST		
D8 to D12	PB treatment (1.5 mg/kg/day, 5 ml/kg, *p.o.*) then PCA test (30 min later)		
D12	–	–	Sampling of brain areas and blood (ChE activity, corticosterone)
D15 to D19	Training for SEA test	Training for WM	–
D52	Recall of SEA test	Recall of WM	–
D113	Recall of SEA test	Recall of WM	–
D199	Recall of SEA test Sampling of brain areas ($n = 60$) (ChE activity, HRMAS)	Recall of WM	

PCA: pole climbing test; SEA: shock-elicited aggression test; WM: water-maze; HRMAS: high resolution magic angle spinning NMR spectroscopy.

stress conditions. For this, we studied learning performances and aggressiveness over the months following the end of the treatment.

2. Materials and methods

All experimental procedures described below were approved by our ethical committee and conducted in accordance with French regulations for the care and use of laboratory animals.

2.1. Subjects

One hundred and ninety-two 3-month-old male Wistar rats (HARLAN, Gannat, France), weighing 350 ± 4 g were housed four per cage in a controlled environment (12 h light/dark cycle, 21 ± 1 °C) with standard food and water *ad libitum*. They were randomly divided into two equal groups: stressed and non-stressed. Each group was then further divided into two equal subgroups: (1) rats treated with pyridostigmine bromide (PB: 3-dimethylamino carbonyloxy-*N*-methylpyridinium bromide, SIGMA, Saint-Quentin Fallavier, France), and (2) non-treated rats given vehicle (in the same way and same volume as PB). Treatment was given as a single oral dose, 30 min before the beginning of each stress session, from day 1 to day 12 except on days 6 and 7. PB was dissolved in water as vehicle and orally administered. Dosing (total salt weight, 1.5 mg/kg/day, 5 ml/kg) was chosen in order to inhibit 30–40% of plasma cholinesterase activity [84] as recommended for the prophylaxis against chemical warfare agents.

2.2. Study design

The different steps of the experiments are summarized in Table 1.

Experimental stress based on the pole-climbing avoidance (PCA) technique (described below) was carried out daily from day 1 to day 12 (except on days 6 and 7), 30 min after PB treatment. From day 15 to day 199 two other behavioural tests were then applied in two separate experiments (A and B) in order to observe the emergence of possible long-term side effects of PB previously given under repeated stress conditions. In experiment A, 80 animals (20 animals/subgroup) were tested (from D15 to D19, then at D52, D113 and D199) for aggressiveness by using a shock-elicited aggression test (SEA test, described below). In experiment B, 80 animals (20 animals/subgroup) were tested for learning (at the same delays as in experiment A) and memory ability (D52, D113 and D199) by using the water-maze (WM) task (described below). Before each session of WM task and SEA test, all of the rats were placed for 2 min into the pole-climbing box for a recall of the initial stressful context without electric foot shock. At the end of the last session of SEA test (experiment A, day 199), 60 rats (15 animals/subgroup, randomly chosen) were killed by decapitation. Brains were quickly removed, placed on ice and hippocampus, striatum, thalamus and anterior cortex were dissected out to measure cholinesterase (ChE) activity and determine metabolite levels (in hippocampus) as described below. The cerebral areas were immediately frozen in liquid nitrogen and stored at −80 °C until use.

In a third experiment (C) 32 animals (8 animals/subgroup) were used to validate PB dosing paradigm and repeated stress model. At the end of the PCA test (day 12), i.e. just after the stress session, rats were killed by decapitation, blood was immediately collected in heparinized tubes and brain was dissected out as previously described. Samples were used to determine plasma corticosterone (CORT), ChE activity in plasma and red blood cells, ChE activity in hippocampus, striatum, thalamus and frontal cortex.

2.3. Behavioural studies

The experimental groups were staggered equally considering that testing was conducted over several hours (i.e. 9 am to 5 pm). Behavioural tests were performed as follows:

(1) *Modified pole-climbing avoidance test (PCA test)*: The pole climbing avoidance test is an avoidance conditioning technique using an auditory stimulus. It has been classically used as a learning test [43]. In our study, it was modified to be used as a stressful procedure by increasing the length of session (25 min, i.e. 25 theoretical trials instead of 10 fixed trials) and the number of sessions (10 sessions instead of 5).

Each rat of the stressed subgroups was placed in a cage (34 cm × 23 cm × 42 cm; Bionic Instruments, Paris, France) equipped with a non-coated wooden mast (32 cm in height and 3 cm in diameter) hanging from the centre of the lid, a floor grid which transmitted electric shock, a buzzer and an electronic device working in regular cycles. Each trial lasted 1 min and included: a rest phase (40 s), a sound (the conditioning auditory stimulus, 300 Hz for 4 s), an interval (1 s) and an electric shock (1 mA for 15 s). The rat could escape or avoid the shock by climbing up the mast, which disconnected the device. The device was connected again only when the animal climbed down. So when the animal climbed down, the cycle was reset to the starting point of the next trial, i.e. the beginning of the rest phase.

Four response options may occur:

1. *Fail escape*: The rat undergoes the shock for 15 s and does not climb up the mast; the rat is not conditioned. The next trial began with the rest phase.
2. *Escape*: The rat undergoes the shock for a few seconds, then climbs up the mast; it acquires the escape response by establishing a relationship between the mast and the cessation of the shock. By climbing up the mast the animal interrupts the program which returns to the original phase, i.e. the rest phase, as soon as the animal climbs down the mast.
3. *Avoidance*: The rat climbs up the mast as soon as it hears the sound, then climbs down. The rat acquires the avoidance response by establishing a time relationship between the sound and the shock. It is an anticipated response: the animal anticipates the shock at a precise moment, i.e. the moment when the sound is produced.

In these three options, the trial is numbered.

4. *Intertrial responses*: The rat climbs up and down the mast at any time, i.e. it generalizes and partially extends the avoidance response. It seems to be rewarded because the device is disconnected then promptly connected again, so the trial is reinitialized to the starting point, i.e. the beginning of the rest phase. If the rat climbs up before the sound, the trial is not numbered. So, in this case, the number of trials per session is decreased.

One 25 min daily session (i.e. 25 theoretical 1-min trials) was applied 30 min after PB or vehicle administration, from day 1 to day 5 then from day 8 to day 12. Non-stressed rats were also placed in the PCA box but without applying the electric foot shock (they explore the box but do not climb up the mast spontaneously). The number of trials per session was recorded.

(2) *Shock-elicited aggression test*: This test was used to assess early and late aggressiveness in rats (experiment A). It is a robust test of inescapable shock-induced aggressiveness previously described by Ulrich and Azrin [82]. One pair of rats (one stressed and one non-stressed from the same treatment-subgroup) was placed daily for five consecutive days (from day 15 to day 19) in a box (50 cm × 25 cm × 25 cm, Bionic Instruments, Paris, France) which allowed animals to move freely without touching one another. Electric inescapable foot shocks (1.5 mA for 10 s) were delivered at the floor grids every 50 s for 20 min using an electronic device (Campden Instruments, Sileby, England). As electric foot shocks begin, the two partners immediately display aggressive behaviour such as tooth-chattering, threat-posturing, boxing position, attacking, leaping