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Pharmacology, Biochemistry and Behavior 82 (2005) 125 – 132

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Stress adversely affects efficacy of physostigmine–scopolamine pretreatment against soman in guinea pigs

Ingrid H.C.H.M. Philippens [a,b,*], Marloes J.A. Joosen [a], Raymond A.P. Vanwersch [a]

[a] *TNO Prins Maurits Laboratory (TNO-PML), Research Group Medical Countermeasures, P.O. Box 45, 2280 AA Rijswijk ZH, The Netherlands*
[b] *Utrecht University (UU), Department of Psychopharmacology, Utrecht, The Netherlands*

Received 30 November 2004; received in revised form 13 June 2005; accepted 12 July 2005
Available online 26 August 2005

Abstract

During military operations, soldiers may be exposed to mixtures of chemicals and to physical, emotional and psychological stress factors, which all may influence efficacy of any treatment, including the nerve agent pretreatment regimen. The purpose of this study was therefore to investigate the influence of chronic intermittent, variable, unpredictable and uncontrollable stress conditions on the side effects and therapeutic efficacy of the combination of physostigmine (0.025 mg/kg/h) and scopolamine (0.018 mg/kg/h) as a pretreatment against 2 × LD50 soman intoxication in guinea pigs. Stress during pretreatment led to an increase of motor activity and an increase of power in the EEG delta2 frequency band. After chronic stress, exposure of pretreated animals to 2× LD50 soman resulted in more severe intoxication symptoms, a more persistent effect on the startle response, and considerable more severe and persistent effects on the EEG power-spectrum, indicating irreversible brain damage.

Keywords: Organophosphate; Soman; Pretreatment; Stress; Physostigmine; EEG; Behavior

1. Introduction

Growing evidence supports the hypothesis that stress during military operations can evoke side effects and impair efficacy of medical treatment. For example, soldiers having served in the Persian Gulf War, often under stressful conditions, are suffering from symptoms with an unidentified cause (Gulf War Syndrome). Pretreatment with pyridostigmine bromide (PYR), in combination with other drugs, under stressful conditions might have contributed to the occurrence of this syndrome. First of all, stress itself might be an important factor in evoking central effects. Stress induces a prolonged corticosterone secretion that leads to a reduction of hippocampal corticosteroid receptors. This decrease of receptors influences a.o. the cholinergic system in the hippocampus. For example, the hippocampal acetylcholine (ACh) release seems to be increased after stress (Mark et al., 1996), probably facilitating occurrence of cholinergic side effects. Furthermore, it is known that stress can change the kinetics of the PYR pretreatment and, therefore, affect the protective ratio and evoke the appearance of side effects (Friedman et al., 1996).

During operation Desert Storm soldiers were given a sign-free dose of PYR, one tablet every 8 h. Nevertheless, peripheral and central side effects were recorded (Keeler et al., 1991), probably due to enhanced passage across the blood–brain barrier (BBB) induced by stress (Friedman et al., 1996). However, in operation Desert Storm nine cases of PYR self-poisoning were encountered. These individuals only suffered from peripheral cholinergic symptoms such as abdominal cramps, diarrhea, hypersalivation and blurred vision, whereas no central effects were recorded (Almog et al., 1991). This finding strengthens the idea that during the operation a combination with other factors, like stress, may play a role in the appearance of side effects and may even influence pretreatment efficacy. This emphasizes the need for considering protective efficacy and side effects of a

* Corresponding author. TNO Prins Maurits Laboratory (TNO-PML), Research Group Medical Countermeasures, P.O. Box 45, 2280 AA Rijswijk ZH, The Netherlands. Tel.: +31 15 284 3048; fax: +31 15 284 3963.
E-mail address: philippens@pml.tno.nl (I.H.C.H.M. Philippens).

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doi:10.1016/j.pbb.2005.07.018

pretreatment not only under standard ("stress-free") but also under stressful conditions.

Pretreatment with physostigmine (PHY) has proved to be very effective against sarin or soman intoxication (Leadbeater et al., 1985). Furthermore under standard test conditions, PHY protects more effectively against soman intoxication than PYR in guinea pigs (Harris and Stitcher, 1984; Solana et al., 1990). This is probably due to the ability of PHY to cross the BBB much more easily than PYR. For the latter reason, PHY was the compound of choice in the present study. To prevent undesirable side effects due to central AChE inhibition by PHY the pretreatment was combined with the muscarinic receptor antagonist scopolamine (SCO, 0.018 mg/kg/h). The effects of stress on side effects of subchronic PHY (0.025 mg/kg/h) combined with SCO pretreatment and its efficacy against soman intoxication were determined in guinea pigs in a therapeutically relevant dose (Gall, 1981; Philippens et al., 1996). Emotional-, physical- and psychological stress were chosen to represent military conditions. Behavioral and neurophysiological parameters were used to elucidate the severity of side effects produced by the combined PHY + SCO pretreatment under prolonged stress, and the influence of stress on the efficacy of pretreatment against soman-induced incapacitation.

2. Methods

2.1. Animals

Male Dunkin–Hartley albino guinea pigs CrL:(HA)BR (Charles River) with an initial body weight of 400–450 g were used. The animals were housed individually in Makrolon type IV cages. The ambient temperature was regulated between 20 and 22 °C. Relative humidity was monitored and kept over 50%. Food and water were always available. The experiments described received prior approval from the Ethical Committee on Animal Experimentation of TNO.

2.2. Drugs

Physostigmine (eserine) and scopolamine bromide were obtained from Sigma (St.Louis, U.S.A.). Atropine Sulphate was obtained from ACF (Amsterdam, The Netherlands). Soman (*O*-pinacolyl methylphosphonofluoridate) was synthesized at the Prins Maurits Laboratory of TNO. Other chemicals used were of standard purity.

2.3. Study design

In this study the side effects of pretreatment with PHY and SCO and its efficacy against soman intoxication were examined under stress circumstances. To this end, behavioral and neurophysiological assessments were carried out. Sixteen guinea pigs were trained in a conditioned learning task, the shuttle-box, after which electrodes for the measurement of electroencephalogram (EEG) were fitted. In order to obtain control values, body weight, plasma cortisol level, shuttle-box performance, startle response, exploration activity in the 'Open Field' (OF) task and EEG were registered. Subsequently, two matched subgroups of 8 animals each were formed, based on equal control values in the neurophysiological and behavioral parameters. The animals of one group (non-stress group) were handled by the standard procedures, whereas the animals of the other group (stress group) were exposed to eight weeks of intermittent, variable, unpredictable and uncontrollable stress.

Once a week the shuttle-box performance, the startle response, the EEG, and the body weight were registered. Every other week the animals were tested in the OF task and blood samples were collected for measuring the plasma cortisol level.

After six weeks of stress induction, pretreatment started by implanting Alzet® osmotic mini-pumps containing PHY and SCO in all animals. During the pretreatment period, exposition to daily stress occasions of animals in the stress group continued. All animals were tested 3 times in the behavioral and neurophysiological read-out systems during the latter period. At day 11 of the continuously administered pretreatment all animals were intoxicated with 2× LD50 soman (49 μg/kg s.c., Gordon and Leadbeater, 1977). One minute after the soman injection the animals received a post-intoxication therapy with AS (0.36 mg/kg i.m.). The osmotic pumps were not removed.

To determine the efficacy of combined PHY and SCO pretreatment in the presence of stress factors, soman-induced post-intoxication incapacitation was investigated. Post-intoxication symptoms, such as hypersalivation, tremors and convulsions were observed. This was followed by measuring the behavioral and neurophysiological parameters after the intoxication symptoms became less severe, 2 h after soman intoxication and repeated at 24, 48, 72 h and 1 week after soman intoxication.

2.4. Stress induction

For eight weeks, animals from the stress group received stress factors for 5 consecutive days, with a resting period of 2 days. Duration of the daily stress factors was 30 min. They consisted of cold stress (30 min in a refrigerator), psychological stress (3 foot-shocks with an interval of 10 min), physical stress (3 times 2 min swimming task with a 10 min interval or 10 min exercise in the running wheel) or emotional stress (placing the animal in an unfamiliar territory for 30 min). To prevent familiarization, each animal from the stress group was exposed to one random stressor at a random time point during the day.

2.5. Determination of cortisol plasma levels

Plasma cortisol levels were measured every other week. Blood samples were collected before stress induction, 15 and

60 min after stress induction. Approximately 60 μl blood was taken from the ear vein and mixed with heparin, followed by centrifugation for 8 min at 2000 *g*. Supernatants were stored at −20 °C until determination of plasma cortisol level, within 10 days from sampling, using a cortisol-kit of ICN. Briefly, 25 μl of plasma was applied in antibody-coated tubes. Subsequently, 0.5 ml of a solution with ^{125}I-cortisol was applied, which binds in competition with the plasma cortisol. This was followed by separation of bound and unbound radioactivity and counting of bound radioactivity.

Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.55 μl/h (Model 2002, Alza Corp., Palo Alto, USA) were used to deliver either vehicle (20% propylene glycol, 10% ethanol and 70% glacial acidified distilled water) or the PHY (0.025 mg/kg/h) and SCO (0.018 mg/kg/h) combination, dissolved in vehicle. This dose of PHY offers the recommended blood-AChE inhibition of about 35%. SCO (0.018 mg/kg/h for a period of ten days) leads to a SCO plasma concentration of 45 nM, which was comparable with the level found after a single s.c. injection of 0.1 mg/kg SCO (43 nM). This SCO plasma concentration caused no side effects on behavior, and antagonized PHY-induced side effects (Philippens et al., 1992, 1996, 1998). The concentrations of PHY and SCO were based on the estimated weight of the animals one week after implantation based on the normal growth curve for guinea pigs in our laboratory. The pumps were implanted subcutaneously on the backs of the animals under ketamine HCl (80 mg/kg i.m., Nimatek®, Eurovet Animal health, Bladel, The Netherlands) and acepromazine (0.5 mg/kg s.c., Vetranquil®, Sanofi sante BV, Maassluis, The Netherlands) anesthesia. The wounds were sutured with wound-clips and disinfected with Nobecutane® wound spray.

2.6. Behavioral tests

2.6.1. Shuttle-box performance

An automated two-way shuttle-box, consisting of two equal compartments of 23 × 23 × 23 cm with rounded corners connected by a photo-cell-guarded gate, was used. The animals had to learn how to avoid a stream of air (about 6 l/s, air tube diameter 1 cm), the unconditioned stimulus (UCS), aimed at their fur within 10 s after presentation of a sound stimulus, the CS. During each trial the CS was followed by the UCS after 10 s. An avoidance response (shuttle behavior during the conditioned stimulus time) was registered as correct avoidance response (CAR) and an escape response (shuttle behavior during the unconditioned stimulus time) was called an escape. Voluntary shuttling between trials was classified as inter-trial response (ITR). During the daily training and test sessions the animals received 20 trials at random inter-trial intervals of 20–30 s. Only animals that reach the criterion of 80% or more CARs after training were used in the experiments (Philippens et al., 1992). The number of CARs was used to express the active avoidance performance.

2.6.2. Auditory startle response

Animals were exposed to 20 auditory startle pulses (120 dB, 10 kHz, 20 ms) while standing with their hind paws on a platform in a vertically mounted PVC-tube (diameter 7 cm, length 16.5 cm). The startle response of 200 ms duration was measured by a transducer connected with the platform, registering the force exerted by the animal upon presentation of the stimulus. An AD converter of an IBM-compatible PC digitized the responses. The area under the curve (AUC), amplitude and latency of the startle response were registered and used to express the motor reaction of the startle reflex.

2.6.3. Open Field task

The Open Field (OF) task is used to measure parameters of spontaneous behavior, like loco-motor activity and exploration in a quantitative way (Tanger et al., 1978).

The OF consisted of a black field of 100 × 100 cm, with 25 cm high enclosing walls. A black grating covered the top of this box. The test room was homogeneously illuminated (about 100 lx) with a background noise of 55 ± 5 dB. A video camera was placed above the OF for registering movement patterns of the white animal in the black area during a 10 min session. The moving patterns were downloaded into a computer. The following parameters were studied: 1) the distance run, 2) the time spent in the "inner field", a 60 × 60 cm virtual area in the centre of the field. The remaining part of the field was defined as "outer field", 3) the number of crossings from outer to inner field, and 4) the number of times the guinea pig changed corners. Corners were defined as virtual squares of 20 × 20 cm in each corner of the field. All parameters are expressed in a cumulative fashion.

2.6.4. Neurophysiological (EEG) measurement

Under ketamine HCl (80 mg/kg i.m., Nimatek®, Eurovet Animal health, Bladel, The Netherlands) and acepromazine (0.5 mg/kg s.c., Vetranquil®, Sanofi sante BV, Maassluis, The Netherlands) anesthesia a silver electrode was placed into a small hole in the skull, 3 mm lateral to the sutura sagitalis and 8.5 mm caudal from the sutura fronto-parietalis, leaving the dura mater intact. A reference electrode was placed over the nasal cavity. Both electrodes were connected with a plug and fixed on the skull with dental cement. During the test, the animals were immobilized in a vertically mounted PVC tube (as for the startle response) and a transmitter was connected to the plug for telemetric registration of the EEG. All EEG signals were amplified (50,000×), filtered (between 0 and 30 Hz) and fed into an AD converter of an IBM-compatible PC; sampling frequency was 50 Hz.

Fast Fourier transformation (FFT), to obtain power spectra, was performed from 5 randomly chosen EEG epochs of 12 s out of a total recording time of 5 min. The obtained power spectra of the guinea pigs were averaged per group and subdivided into 7 frequency classes (delta1: 0.8–

2, delta2: 2–3.5, theta1: 3.5–5.5, theta2: 5.5–7.5, alpha1: 7.5–10, alpha2: 10–12.5, beta: 12.5–25 Hz). The total powers (V^2) of the different frequency classes were used for the evaluation of the brain activity.

2.7. Post-intoxication symptomatology

Upon soman intoxication, the following symptoms were scored: 1) chewing: clear chewing-like movement of the guinea pig in which the entire head is involved; 2) hypersalivation: extensive drooling; 3) mild tremor: slight shivering; 4) severe tremor: intense shivering, the entire body is involved; 5) convulsions: involuntary tensed movement in which the entire body is involved, during which the animal is refractory to stimulatory impulses and 6) dyspnoea: low respiratory rate and heavy breathing, often accompanied with dark eyes.

The symptoms were scored every other minute during the first hour, followed with 15 min intervals during the next 6–8 h. More than one symptom can be scored at the same time. The severity of a symptom was calculated as the percent of total scoring hits of the animals in which the symptom was observed.

2.8. Statistics

For statistical analysis of the behavioral and neurophysiological tests an analysis of variance (ANOVA) followed by a Newman–Keuls post hoc test or an unpaired *t*-test with Welch's correction was used. For the symptomatology after soman intoxication a Fisher exact probability test and an unpaired *t*-test with Welch's correction was used. In all tests p values <0.05 were considered significant.

3. Results

In the present study, the effect of stress alone, its effect on the appearance of side effects during PHY and SCO pretreatment, and on the efficacy of the pretreatment in preventing the toxicity of 2× LD50 soman were tested.

3.1. Cortisol levels

The intermittent, variable, unpredictable and uncontrollable stress used in this study, induced a strong significant increase of the plasma cortisol levels measured after 15 and 60 min after the stress induction (Fig. 1). There was no difference found between 15 and 60 min after stress induction on the increase of plasma cortisol.

3.2. Shuttle-box

During the first four weeks of stress induction, all animals from the stress group showed a significant higher number of ITRs in the shuttle-box (ITR non-stress group: 2.21±0.34, ITR stress group: 7.63±0.88; $p<0.05$, see Fig. 2). However, after the first three weeks of stress induction the stressed animals reacted similar to the non-stress animals in the shuttle-box. During the following pretreatment period, no effects of stress were found in the shuttle-box.

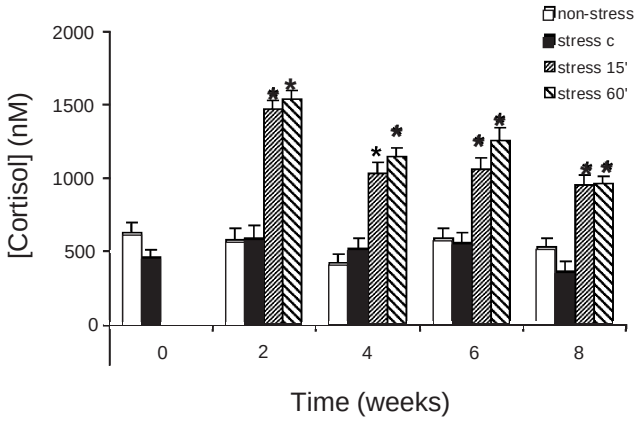

Fig. 1. Plasma cortisol levels (nM) measured in stressed guinea pigs ($n=8$) and in non-stressed guinea pigs ($n=8$) after 2, 4, 6, and 8 weeks of stress induction. During weeks 7 and 8 all animals were also pretreated with PHY and SCO. The cortisol values of the stressed animals were measured before (stress c), 15, and 60 min after stress induction. All values after stress induction were significantly increased (ANOVA and Newman–Keuls post hoc test, *: $p<0.05$).

Upon soman intoxication, all animals of both groups were physically able to perform the task in the shuttle-box; they showed a normal ITR activity after soman intoxication. However, the number of CARs of animals in both groups had significantly decreased from 96.9±1.3 to 28.8±8.8 in the stress group and from 93.8±2.3 to 38.8±8.9 in the non-stress group. No significant difference was found between the two test groups. Twenty-four hours later their performance had slightly improved, but the effect remained present during one week after soman.

3.3. Open Field

The activity in the OF task was not affected by stress or pretreatment alone (Fig. 3). On the other hand, during pretreatment with PHY and SCO under the stressful conditions, the entries corners and inner field in the OF task were significantly increased (Fig. 3; $p=0.028$ and $p=0.022$, respectively). Also a slight but insignificant increase in distance run was found in the stressed animals. Stress had no effect on the efficacy of pretreatment against soman intoxication in this task (Fig. 3).

3.4. Startle response

The results of the startle response are shown in Fig. 4. AUC and amplitude (not shown) showed a tendency towards a small but not significant increase induced by stress. Pretreatment with PHY and SCO did not affect AUC in both groups.

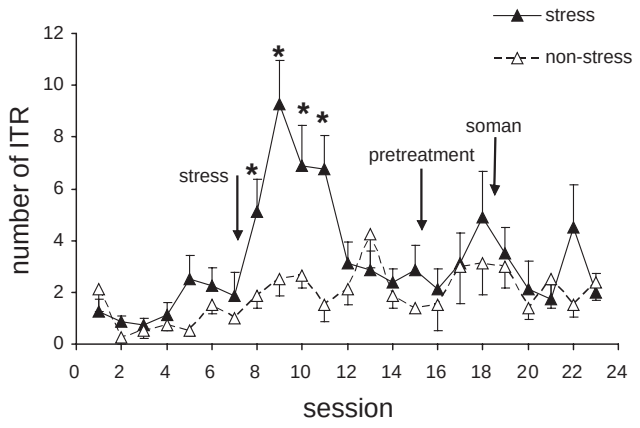

Fig. 2. Number of inter-trial responses measured in stressed guinea pigs ($n=8$) and in non-stressed guinea pigs ($n=8$) during the training period (session 1 to 7), after the first 3 weeks of stress induction (sessions 8–11), after the second 3 weeks of stress induction (sessions 12–15), during pretreatment (sessions 16–18), and after soman intoxication (sessions 19–23). The number of ITRs during the first three weeks of stress induction was significantly increased (ANOVA and Newman–Keuls post hoc test, *: $p<0.05$).

After 2× LD50 soman intoxication, both test groups showed an increase of the startle response, with a more persistent character in the stress group.

3.5. EEG

The EEG power spectrum was unaffected during stress induction. During pretreatment however, an increase was found on the power of the delta2 band (2.0–3.4 Hz) in the stress situation. The baseline value was 1478±162 V^2 ($n=8$), which was during the pretreatment period significantly increased to 2116±205 V^2 in the stress group ($n=8$, ANOVA with Newman–Keuls correction: $p<0.05$).

In all animals of both groups an increase was found on the delta2 band of the EEG 2 h after soman intoxication (see Fig. 5). The animals of the stress group showed also effects on the delta1 and theta1 bands (ANOVA with Newman–Keuls post hoc: increase $p<0.01$ versus decrease $p<0.05$). The latter effects in the stressed animals were more persistent whereas the effects found in the non-stressed animals had already disappeared one day later. Furthermore, the stressed animals showed a significant persisting decline of the power of the theta1, which is an indication for the mental status of the animals.

3.6. Symptomatology

All animals in both groups (stress and non-stress) survived the 24 and 48 h criteria after intoxication with 2× LD50 soman. The post-intoxication symptoms observed following soman intoxication are summarized in Table 1. Stress adversely affected the post-intoxication symptomatology: the appearance and severity of symptoms were significantly different between stress versus non-stress (two-way ANOVA, $p=0.004$).

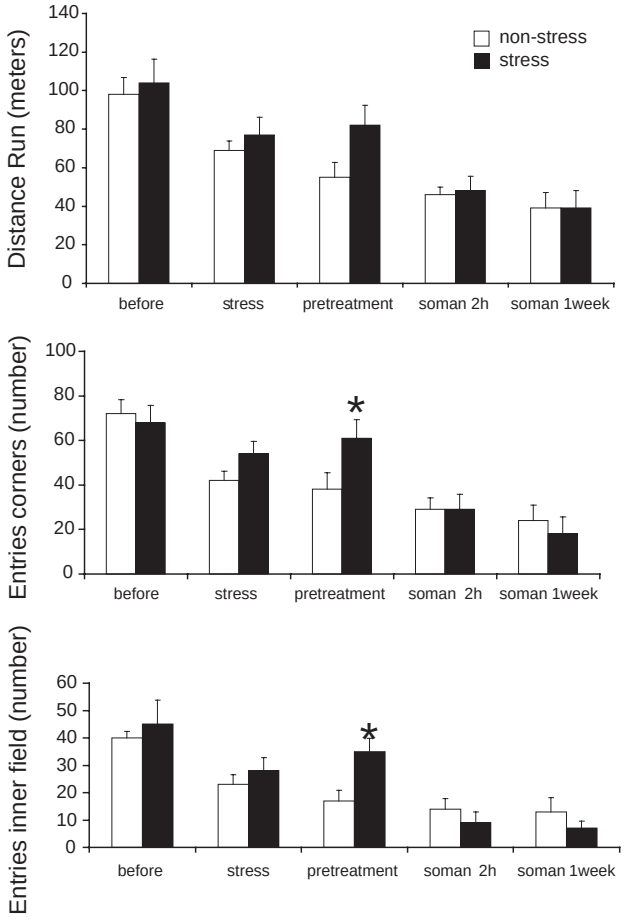

Fig. 3. Behavior in the OF measured in two different groups ($n=8$/group), non-stress or stress, before stress induction, after 6 weeks of stress induction (stress group), after 10 days of PHY and SCO pretreatment, 2 h and 1 week after 2× LD50 soman. The distance run was expressed as the cumulative meters walked during the 10 min session (mean±SEM). The entries corners or inner field was expressed as the cumulative number of entries in these virtual areas during the 10 min session (mean±SEM). *Significantly different from non-stress. ($p<0.05$).

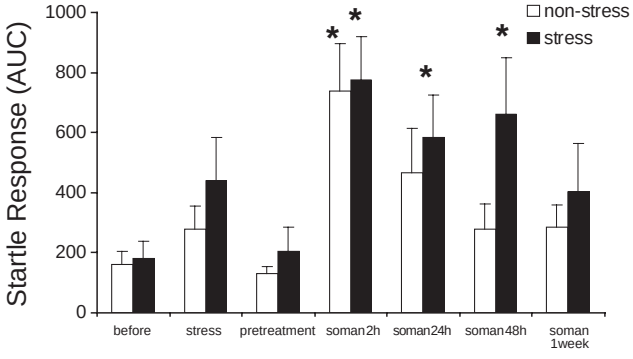

Fig. 4. Area under curve (AUC) of the startle response of 200 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects before, after 6 weeks of stress induction, after 10 days of PHY/SCO pretreatment and after soman intoxication (2, 24, 48 h and 1 week) in non-stressed and stressed guinea pigs ($n=8$/group, mean±SEM). *Significantly different from baseline value (before) ($p<0.05$).

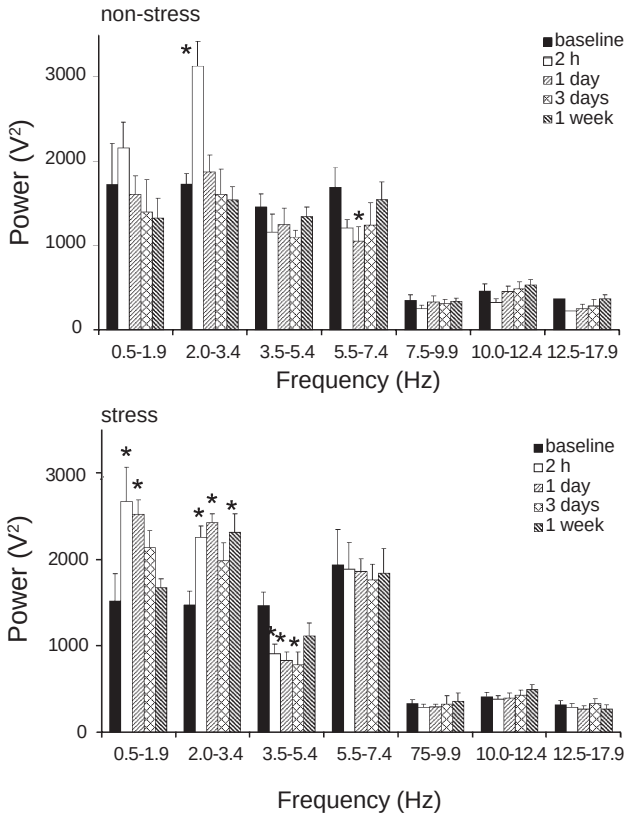

Fig. 5. Power spectrum of different frequency bands of the EEG of standard (no-stress) or stress exposed guinea pigs (V^2, mean ± SEM, $n = 6-8$ animals per test session), before any treatment (baseline), and after soman intoxication (2× LD50) in PHY + SCO pretreated animals (2 h, 1, 3, and 7 days). *Significantly different from baseline value ($p < 0.05$).

Three out of 8 animals in the non-stress group showed convulsions, 3 other animals showed severe tremor as most severe symptom, whereas the remaining 2 animals only showed mild tremor. In the stress-exposed group on the other hand, 2 animals exhibited convulsions as most severe symptom, and 3 animals even suffered from dyspnoea. All stressed animals showed at least severe tremors as a consequence of soman intoxication.

Four days after soman (day 15) one animal of the non-stress group died and one day later (day 16) one animal from the stress group died. The latter animal showed the most and most severe symptoms upon soman intoxication.

4. Discussion

The therapeutic efficacy of a combined pretreatment of PHY and SCO against 2× LD50 soman was investigated in guinea pigs under chronic intermittent, variable, unpredictable and uncontrollable stress conditions. Three aspects were evaluated using several behavioral and neurophysiological parameters: the effects of the stress procedure itself, the appearance of side effects during pretreatment, and the protection against post-intoxication incapacitation after intoxication by 2× LD50 soman.

4.1. Effects of stress induction

During stress induction, the behavior in the shuttle-box was affected. The two-way shuttle-box is a task sensitive for emotional changes, because of the conflict situation in which the animals have to initiate a response towards a location where they have previously experienced aversive events (Clincke and Werbrouck, 1993). Only during the first three weeks of the eight-week period of stress induction, the stressed animals showed an increased number of ITRs. This could be explained as an enhanced activity of the stressed animals. However, in the OF task no increase of the distance run (a measurement of activity) was observed. Presumably, the increase of ITR was due to an increased alertness.

Stress probably induces aversive emotions, such as fear and anxiety. These emotional states have been shown to influence the startle response (Lang et al., 1990; Davis et al., 1993). A small but insignificant increase of the startle reaction was found during the six weeks of stress induction, which had disappeared after starting with the PHY and SCO pretreatment, pointing to emotional, alertness enhancing effects of stress.

4.2. Effects of stress on side effects during pretreatment

During the 11 days of continuously applied pretreatment of PHY and SCO no effects were found in any of the test systems used under the standard conditions.

Table 1
Post-intoxication symptomatology after 2× LD50 soman and AS in continuously PHY and SCO pretreated guinea pigs under standard conditions (non-stressed) or stress-full conditions (stressed) expressed as the percent of the total scoring

Non-stress	Animal number								Total animals	Severity
Symptom	5	9	10	12	13	14	20	21		%
Chewing	8	20	7	21	13	8	0	0	6/8	12.7±2.6
Hypersalivation	0	0	0	0	0	0	0	0	0/8	
Mild tremor	25	54	72	10	55	39	23	55	8/8	41.6±17.4
Severe tremor	0	29	17	24	39	31	0	14	6/8	25.5±3.8
Convulsions	0	0	0	62	13	12	0	0	3/8	28.8±16.6
Dyspnoea	0	0	0	0	0	0	0	0	0/8	
Total symptoms	2	3	3	4	4	4	1	2		2.9±0.4

Stress	Animal number								Total animals	Severity
Symptom	3	6	8	15	16	17	19	22		%
Chewing	12	58	70	54	31	52	29	64	8/8	16.3±4.0
Hypersalivation	0	0	0	0	14	0	18	0	2/8	16.1[a]±1.8
Mild tremor	65	53	49	43	23	43	21	50	8/8	43.3±5.2
Severe tremor	41	34	18	36	31	30	18	32	8/8	30.0±2.9
Convulsions	31	0	0	14	37	11	36	0	5/8	25.7±5.5
Dyspnoea	4	0	0	0	40	0	4	0	3/8	15.9[a]±12.1
Total symptoms	5	3	3	4	6	4	6	3		4.3[a]±0.5

Total animals: number of animals in which the symptom was observed. Total symptoms: total number of different symptoms observed in the animal. Severity of the symptom expressed as percent ± SD of total scoring hits of the animals in which the symptom was observed.
[a] Significantly different from non-stress group ($p < 0.05$).

This is in accordance with our previous observation that startle response, neurophysiological parameters and performance in the shuttle-box were not affected by the same pretreatment in guinea pigs (Philippens et al., 1998). In the current study, the effect of this pretreatment on OF behavior was investigated for the first time. In the non-stressed animals, pretreatment did not affect this task (Fig. 2), whereas in stressed animals the pretreatment induced side effects in the OF. The type of effects (increase of "entries corners" and "inner field" and slight increase in "distance run") points to increased activity. Such increased activity is presumably due to stimulation of cholinergic receptors induced by the increase of ACh induced by AChE-inhibition after PHY and due to the increased ACh release after stress (Mark et al., 1996).

Pretreatment affected the EEG in that an increase of the delta2 band was found. From other studies it is known that cholinergic compounds may affect this frequency band (Riekkinen et al., 1991; Wolthuis et al., 1991; Timofeeva and Gordon, 2001). These effects are presumably the result of indirect effects of elevated ACh levels due to the inhibition of AChE (Timofeeva and Gordon, 2001).

4.3. Effects of stress on efficacy of the pretreatment

4.3.1. Symptomatology and survival

The high protection rate found in this study is in accordance with our previous study, in which the addition of SCO to the pretreatment or addition of AS post-intoxication therapy synergistically enhanced protection against soman-induced lethality (Philippens et al., 2000).

The difference between stressed and non-stressed animals was most pronounced by the post-intoxication symptoms. All 8 stressed animals showed severe tremors and 5 of them convulsions compared to 6 and 3 animals, respectively, in the non-stressed group. Dyspnoea was only found in the stressed animals, a symptom which was previously found only in animals without a post-intoxication therapy with AS (Philippens et al., 2000). Therefore, these effects in a stressful situation could be the result of a combination of an increase of ACh release and AChE-inhibition or the system becoming more susceptible to ACh. It has been reported that inescapable stress significantly increased ACh release in hippocampus and prefrontal cortex (Mark et al., 1996). Additionally, the number of muscarinic receptors in several brain areas was significantly increased upon chronic stress (Gonzalez and Pazos, 1992). Increased numbers of muscarinic receptors and increased release of ACh as a result of stressful experimental conditions might explain the more severe symptoms observed in the present study. An increase of BBB permeability caused by stress, facilitating entrance of PHY and soman into the brain, could also have contributed to the decreased therapeutic efficacy. This is supported by Dvorska et al. (1992) and Skultetyova et al. (1998), who showed that even short-lasting immobilization stress led to increased permeability of the BBB.

4.3.2. Post-intoxication incapacitation

Non-stressed animals were best protected against the soman-induced incapacitation. Although a large increase of the startle amplitude and AUC was found in both groups, these effects were more persistent in the stress group. In a previous study it was clarified that rather direct effects on nicotinergic receptors than AChE inhibition were involved changing the startle amplitude (Philippens et al., 1997). Besides its AChE-inhibiting effect, soman exhibits direct effects on nicotinergic receptors (Bakry et al., 1988), which is also the case for PHY (Albuquerque et al., 1984; Sherby et al., 1985). Another explanation for the more persistent effect on the startle response in the stress group, might be an altered release of 5-HT upon stress (Davis et al., 1982), or the serotonine-induced increased permeability of the BBB under stress conditions (Sharma and Dey, 1981).

The most pronounced effect was shown on the EEG. In the stressed animals a persisting increase of the delta1 and delta2 was found besides the decline in theta1 power. These effects are indicators of irreversible brain damage (Carpentier et al., 2001). It seems that the returning of the power from the delta band to its baseline level is not correlated with the recovery of brain damage but with cleaning of necrotic tissue. This means that after the delta activity has normalized, brain damage is still present (McDonough, Jr. et al., 1998). Slow theta is associated with relaxed waking and grooming activity (Kramis et al., 1975; Vanderwolf and Baker, 1986) and is atropine sensitive (Stewart and Fox, 1989a,b). Down-regulation of muscarinic receptors, by soman-induced AChE inhibition (Gazit et al., 1979; Costa et al., 1982; van Dongen and Wolthuis, 1989) and enhanced ACh release by stress (Mark et al., 1996), may lead to this decline of the theta1 power.

From the present experiments it can be concluded that stress indeed influences the efficacy and side effects of the pretreatment. Although PHY already easily penetrates into the brain because of its structure, stress rather influences the appearance of undesirable side effects of the pretreatment. After 2× LD50 soman, symptoms are much worse in stressed than in non-stressed animals. Stress even increased the probability of irreversible brain damage. This may be due to either changes in the BBB, or cholinergic effects, such as increased release of ACh and changes in the number of muscarinic receptors. Hence, the present study indicates that stress should be considered when testing the efficacy of medical (pre)treatment of OP intoxication.

Acknowledgements

The authors would like thank Bas Groen and Marjan Jongsma for their excellent technical assistance.

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