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An acute exposure to a sub-lethal dose of soman triggers anxiety-related behavior in guinea pigs: Interactions with acute restraint

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Abstract

In this study, we tested the hypothesis that a single exposure of guinea pigs to sub-lethal doses of soman triggers anxiety-related behavior that is modifiable by acute stress. Prepubertal male guinea pigs were subjected to one of the following treatments: (i) saline (0.5 ml/kg, sc), (ii) soman (0.6x or 0.8xLD₅₀, sc), (iii) saline followed 30 min later by 2-h restraint, or (iv) soman followed 30 min later by 2-h restraint. Behavior of the animals was examined two and three months later in a large open field and in the elevated-plus-maze. Animals that had been exposed to restraint stress alone or soman alone showed decreased exploratory activity when tested in the open field with bare floor at 20–30 Lux. Total distance traveled and distance traveled in the center of the field were shorter for animals that were exposed to either restraint stress or soman than for saline-injected animals. In addition, animals challenged with soman or restraint stress remained immobile for a longer time in the open field than did saline-injected guinea pigs. Performance in the elevated plus maze test revealed that exposure of guinea pigs to soman or restraint stress decreased their number of entries and the time spent in the open arms of the maze (measures of anxiety) and reduced their overall locomotor activity. Soman exposure and restraint stress cancelled out each other's effect on locomotion, while only attenuating one another's effect on anxiety-related behavior. It is concluded that a single exposure to sub-lethal doses of soman triggers long-lasting anxiogenesis and decreased locomotor activity and that acute stress modifies the magnitude of these effects.

Keywords

Anxiety; locomotion; open field; elevated plus maze; organophosphorus compounds

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1. Introduction

Nerve agents, including soman, sarin, tabun and VX, are organophosphorous compounds (OP) chemically related to, but far more toxic than the majority of pesticides used worldwide in agriculture and households. There have been reports of the catastrophic use of some of those agents as weapons of mass destruction in wars and in terrorist attacks [1].

The acute toxicity of nerve agents is well documented and results primarily from irreversible inhibition of acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine (ACh). Immediately following an exposure to toxic doses of these agents, overactivation of muscarinic and nicotinic receptors by excess ACh, both within and outside the central nervous system (CNS), triggers a syndrome characterized by miosis, blurred vision, dyspnea, cough, nausea and vomiting, headache, muscle weaknesses, muscle fasciculations, decreased consciousness level, and convulsions. High doses result in death from respiratory failure, involving both central and peripheral mechanism [2]. Acute poisoning with soman is especially difficult to treat because of the rapid aging of the soman-inhibited AChE that renders the enzyme refractory to reactivation by oximes [2].

The terrorist attack with sarin in the Tokyo subway in 1995 represented the largest exposure of a civilian population to nerve agent. Twelve out of the possible 5,500 victims of the attack died. It is reported that only 16% of the 640 patients admitted to one of the hospitals presented with moderate signs of acute intoxication requiring immediate therapeutic intervention; less than 1% of those patients had severe signs of acute intoxication requiring intubation [3]. However, six months to seven years later, increased incidence of posttraumatic stress disorder and chronic cognitive impairments were reported among the victims of the sarin attack who did not necessarily present with signs of acute toxicity at the time of the incident [4,5]. Cognitive dysfunctions have also been observed in rescue team members three years after their initial exposure to sarin at the catastrophe site [6]. Finally, it has been suggested that the exposure of soldiers to sub-lethal doses of nerve agents during the Gulf War may have contributed to the delayed development of a group of clinical manifestations currently referred to as the Gulf War syndrome [7].

Insufficient knowledge of the behavioral effects of sub-lethal doses of nerve agents has made it difficult to unequivocally assign a cause-consequence relationship between an initial exposure to low doses of these agents and specific neurological deficits in humans. Further, in all the circumstances described above, stress is a significant confounding factor. Stress is known to increase acetylcholine release in some brain areas [8] and to aggravate the negative acute effects of an exposure to lethal doses of nerve agents [9]. However, the impact of stress on the behavioral effects of low doses of these agents is not well known.

Immediate and long-term behavioral effects of acute intoxication with nerve agents are well characterized, and seizure activity is believed to be the main underlying cause [10,11]. In contrast, few studies have examined the behavioral effects of a single exposure of rodents to sub-lethal doses of nerve agents [12–14]. Hypolocomotion has been detected in rats at various times (one day to five weeks) following a single exposure to a low dose of sarin or

soman [12,14]. Anxiety-related behavior has also been observed in rats following a single exposure to a low dose of soman [13,14].

The specific aims of the present study were to identify the long-term behavioral effects of a single exposure to sub-lethal doses of soman in guinea pigs, the best non-primate model of OP toxicity [15,16], and to determine how these effects are modified by exposure of these animals to acute stress. Prepubertal male guinea pigs were subjected to different challenge doses of soman (0.6x or 0.8xLD50, sc) and/or a 2-h restraint stress. Two to three months later, locomotor activity and anxiety-related behavior of the animals were evaluated in the open field and in the elevated plus maze. Results presented herein demonstrate that a single exposure of guinea pigs to sub-lethal doses of soman triggers long-lasting anxiety-related effects that are significantly modified by acute stress.

2. Materials and Methods

2.1. Animals

Male Hartley guinea pigs [CrI(HA)Br] were purchased from Charles River Laboratories (Wilmington, MA) and housed in stainless steel cages (60 × 60 × 25 cm), in a climate controlled animal-care unit with constant temperature of $21 \pm 0.5^{\circ}\text{C}$ and a 12-h light/dark cycle. Animals were 28–30 days old (300–320 g) on arrival at the animal-care unit. Food and water were available ad libitum. In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.2. Methodological study

In contrast to rats and mice, guinea pigs like humans and non-human primates express low levels of plasma carboxylesterases – the enzymes that metabolize OP compounds [15,16]. Consequently, the sensitivity of guinea pigs to the toxicity of OP compounds and their responsiveness to OP antidotes resemble those of primates [15,16]. Although guinea pigs have become the rodent species of choice for studies of OP toxicity, they have not been very often used in studies of spontaneous behavior because their high level of anxiety in a novel environment inhibits their exploratory activity. Guinea pigs have even been considered as unsuitable animals for behavioral research [17].

Given the natural ability of guinea pigs to explore their home cages, a methodological study was conducted to determine whether decreasing the anxiogenic effects of the testing environment would disinhibit the exploratory activity of the animals in the open field. In this methodological study, four animals were tested at two months of age in a large open field apparatus. Tests were performed once during each of three consecutive days. The first and the third tests were performed in the open field with the bare floor. The second test was performed in the same apparatus with the floor covered with the same type of paper bedding as used in the home cages. Light intensity in the open field was kept at a 80–100 Lux during all three tests.

2.3. Exposure to soman and/or restraint stress

To examine the effects of soman on spontaneous behavior, guinea pigs received a subcutaneous (sc) injection between the shoulder blades of 0.6xLD50 (16.8 µg/kg) soman (n = 8), 0.8xLD50 (22.4 µg/kg) soman (n = 9), or saline (n = 8) at the age of 35–36 days. After the soman challenge, gross behavior of the guinea pigs was observed continuously for 30 min. In the groups of animals injected with 0.6xLD50 soman or saline, half of the animals were transported to another room with an ambient temperature of $21 \pm 0.5^{\circ}\text{C}$ and placed in Plexiglas restrainers for 2 h. The other half was left undisturbed in the cages. Four of the nine guinea pigs exposed to 0.8xLD50 soman were moved to the room where they were subjected to the acute stress, and the other five were left in their cages. At the end of the 2-h restraint, animals were removed from the restrainers and observed for an additional 2 h. Then, all animals were transported to the animal facility, where they were maintained for the next three months. Two stressed and two nonstressed animals were housed together for each test group, except for the animals challenged with 0.8xLD50, which were housed in groups of four (two stressed and two non-stressed) or five (three stressed and two non-stressed). At 48 h after the restraint session, one of the animals that had shown mild signs of intoxication when challenged with 0.8xLD50 soman was very ill, lost more than 20% of its initial body weight, and had to be euthanized (see Results).

2.4. Behavioral testing

Two months after the injection of soman or saline and/or the acute stress challenge, exploratory and anxiety-related behaviors were assessed in the open field test. Based on the finding that lowering anxiety of the novel environment increased the exploratory activity of the guinea pigs in the open field (see Results), the first test was performed under high-light conditions in an open field apparatus that had the floor covered with paper bedding. Two days later, guinea pigs were tested under low-light conditions in the open field with bare floor. One month later (i.e., three months after the initial injections), guinea pigs were tested again on the open field with bare floor at low-light conditions. High- and low-light conditions refer to light intensities of 80–100 and 20–30 Lux, respectively, within the open fields.

From the day of arrival at the animal-care unit, animals were handled twice-a-week during changing of the home cage bedding and during weighing sessions. On the day of testing, guinea pigs were allowed to acclimate to the testing room for a minimum of 2 h. Following the acclimation time, each guinea pig was placed in the center of the open field and tested for 10 min. In between tests, the open field was washed with 70% alcohol and well ventilated prior to testing the next animal.

Seven days after the second test in the bare open field, animals were tested in the elevated plus maze in the same room and under the same lighting conditions (20–30 Lux) as used for the bare floor open field test. Each guinea pig was placed in the center compartment of the maze, faced toward an open arm, and its behavior was video recorded for 5 min. Entries into a given arm were counted when an animal had all four paws in the arm. Entries to the center zone were counted when at least 75% of animal body with the head was in the zone, because the zone could only accommodate that much of an animal.

2.5. Testing equipment and analysis of open field and elevated plus maze

The open field apparatus consisted of a black, non-glare Starboard plastic box ($120 \times 120 \times 60$ cm). For the purpose of data analysis, a virtual center zone ($40 \text{ cm} \times 40 \text{ cm}$) was delineated 40 cm from the walls. The elevated plus maze was made of wood, painted a graphite color, and consisted of two open arms ($50 \times 14 \times 1$ cm) and two closed arms ($50 \times 14 \times 30$ cm). The apparatus was elevated to a height of 45 cm.

All open field tests were video recorded and analyzed using the ANY-maze Video Tracking System (Stoelting Co., Wood Dale, Illinois). Time immobile was defined as immobility of at least 70% of animal body for at least 5 s. Locomotor activity in the elevated plus-maze was also video taped and analyzed using the ANY-maze software. The number of entries and time spent in arms was scored by an observer.

2.6. Drugs

Soman (O-pinacolyl methylphosphonofluoridate) was obtained from the United States Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). Storage, handling, and disposal of soman conformed to the rules set forth by the United States Army. On the day of the experiments, a vial containing an aliquot (0.2–0.5 ml) of stock solution of soman (1.88–1.90 mg/ml), was diluted with sterile saline to appropriate concentrations and kept on ice for the duration of the experiment. At the end of the experiments, any remaining diluted OP and materials contaminated with OPs were decontaminated with 10% sodium hydroxide before disposal.

2.7. Statistical analysis

Data were analyzed by two- or three-way ANOVA. Results of the open field test were log transformed before analysis. Whenever interactions and/or main effects were found to be significant, pairwise post-hoc comparisons followed by the Bonferroni adjustment were performed.

3. Results

3.1. Acute effects of a single exposure of guinea pigs to a sub-lethal dose of soman and/or 2-h restraint stress

The gross behavior and weight gain of saline-injected guinea pigs that were subjected to a 2-h restraint stress was comparable to that of saline-injected animals that were not placed in the restrainers. Likewise, none of the eight guinea pigs challenged with 0.6xLD₅₀ soman presented any clear signs of intoxication. The four guinea pigs from this group that were subjected 30 min later to a 2-h restraint stress also presented no signs of acute toxicity. In addition, their gross behavior was indistinguishable from that of saline-injected animals, regardless of their exposure to the acute stress.

Four of the nine guinea pigs exposed to 0.8xLD₅₀ presented signs of mild toxicity, specifically facial twitches, chewing and slight hyperlocomotion. Of the five guinea pigs that presented no sign of acute toxicity when challenged with 0.8xLD₅₀ soman, two were subjected to the 2-h restraint stress and three remained undisturbed in the cage. At the end of

the restraint session, these animals exhibited no gross behavior abnormality. Subsequently, their weight gain was comparable to that of saline-injected animals, regardless of their exposure to acute stress (Figure 1). Two of the four guinea pigs that showed signs of mild intoxication in response to 0.8xLD50 soman were also subjected to the 2-h restraint stress 30 min later, whereas the other two were left undisturbed in their cage. In the latter animals, the signs of toxicity subsided within 2–4 h after the initial soman challenge. In contrast, restraint worsened the conditions of one of the guinea pigs that presented signs of toxicity within 30 min of the challenge with 0.8xLD50 soman. This animal had significant muscle weakness when it was removed from the restrainer. During the next 48 h, this animal lost approximately 20% of its body weight, became very lethargic and had to be euthanized.

3.2. Methodological study of the spontaneous behavior of guinea pigs in the open field

Distinct from most rodents used in biological research, guinea pigs respond to novel environments by remaining still for long periods of time. Some possible ways to overcome this problem include: (i) habituating the animals to the test environment and to daily handling [18], (ii) decreasing the anxiogenic effect of the test environment, and/or (iii) using escape from or avoiding of noxious stimulus as a reward [17,19]. Thus, guinea pigs have been shown to perform as well as rats and mice in a T-shaped water maze, where they have to escape from the water onto a hidden or visible platform [17]. Likewise, guinea pigs can be trained to avoid electrical shocks in shuttleboxes [19] and have been shown to avoid anxiogenic stimuli (unfamiliarity, openness and height) in the elevated plus maze [20].

Here, the spontaneous locomotor activity of a cohort of control (untreated) guinea pigs was analyzed in the open field under different conditions. Light intensity was kept at 80–100 Lux, whereas the floor of the field was either bare or covered with the same type of paper bedding as that used in the home cages. On days 1, 2, and 3 the behavior of each guinea pig was recorded for 10 min in the open field with bare floor, paper bedding-covered floor and bare floor, respectively. The animals only exhibited significant exploratory behavior in the open field when the floor of the apparatus was covered with paper bedding (Figure 2). These results support the notion that decreasing the anxiogenic effect of the environment improves the spontaneous exploratory behavior of the guinea pigs. Thus, the experiments below were conducted in the open field with paper bedding-covered floor or in the bare open field under low light illumination, a condition also known to attenuate the anxiogenic effect of a novel environment [21].

3.3. Behavior of guinea pigs in the open field at two and three months after a single exposure to soman and/or acute stress

The first set of experiments was conducted at 80–100 Lux in the open field with the paper bedding-covered floor at two months after the guinea pigs had been challenged with soman (0.6x or 0.8xLD50) and/or exposed to acute stress. Two-way ANOVA revealed no significant main effect of soman or restraint stress on the total distance the animals traveled in the open field, the distance they traveled in the center of the field, or the time they remained immobile (Figure 3A). However, a significant interaction between soman and stress was observed on the total distance traveled and immobility time ($F(2,18) = 6.62$, $p < 0.05$, and $F(2,18) = 6.62$, $p < 0.01$, respectively). Pairwise post-hoc comparisons followed by

Bonferroni's correction indicated that in the absence of stress soman had no significant effect on the exploratory activity of the guinea pigs. In contrast, in the absence of soman, stress significantly decreased the total distance the animals travelled and increased the time they remained immobile in the field (Figure 3A).

The second set of experiments was conducted two days later. The same animal cohort as in Figure 2A was tested under low-light conditions (20–30 Lux) in the open field with bare floor. These same conditions were used to re-test the animals three months after the initial challenge. At 20–30 Lux, control (saline-injected) guinea pigs maintained exploratory activity in the open field with bare floor two or three months after the injection (Figure 3B). Three-way ANOVA with the time of the post-challenge test as a repeated measure factor revealed no significant main effect of soman, stress, or testing time on the total distance the animals traveled in the field or the total time they remained immobile. There was also no significant main effect of soman or stress on the distance the animals traveled in the center of the field. The testing time, however, had a significant main effect [$F(1,23) = 4.45$, $p < 0.05$] on this parameter, with the guinea pigs exploring the center of the arena more at three than at two months after treatment. It has been reported that open-field testing of rodents measures the effects of a given experimental condition on the reaction of the subjects to the stressful, novel environment and on their locomotor activity [22]. It is well accepted that the less anxious the animals are, the more they explore the center of the open field [22]. Therefore, the finding that the guinea pigs presented higher exploratory activity in the center of the field at three than at two months post-challenge suggests that anxiety may decrease as the guinea pigs age.

For all three measured parameters, the interaction between soman and stress was significant [$F(2,36) = 16.01$, $p < 0.0001$; $F(2,36) = 10.84$, $p < 0.001$; and $F(2,36) = 15.50$, $p < 0.0001$, for the total distance traveled, distance in the center, and time spent immobile, respectively]. Because there was no significant interaction of test time with soman and stress, pairwise post-hoc comparisons followed by Bonferroni's correction to determine the significance of the effects of soman and acute stress were performed after collapsing the results obtained at two and three months. In the absence of acute stress, both doses of soman significantly decreased the exploratory activity of the guinea pigs (Figure 3B). In the absence of soman, acute stress also caused significant long-lasting inhibition of the exploratory activity of guinea pigs (Figure 3B). The finding of a significant interaction between soman and acute stress in the absence of significant main effects for either factor suggests that the association of the two factors cancelled each other's effects.

3.4. Behavior of guinea pigs in the elevated plus maze two months after a single exposure to soman and/or acute stress

The elevated plus maze test was performed to confirm whether changes induced by soman and/or stress in the open field behavior of guinea pigs were anxiety related. It is generally accepted that percentages of entries and time spent in the open arms are inversely correlated to anxiety in rodents [23]. Evidence has also been provided that the total number of entries in closed-plus-open arms reflects both the locomotion and the anxiety of rodents, whereas number of entries in closed arms is a measure of locomotor activity [24].

At three months after the treatments of the guinea pigs, the number of entries and the time spent in the open arms, closed arms, and center zone, and the total distance they traveled in the maze elevated plus maze were measured. Results are not presented as % of entries into open arms because of the low activity and relatively long time the animals, specifically those challenged with soman and/or exposed to restraint, spent immobile in the center zone of the maze. One possible reason for these animals to hesitate to enter the closed arms is that their level of anxiety was so high that they exhibited freezing behavior and stayed in the center of the maze, where they were placed.

Significant interactions between soman and stress were observed on the number of entries and the time spent in the open arms [$F(2,18) = 11.78$, $p < 0.001$ and $F(2,18) = 6.75$, $p < 0.01$, respectively], with soman having a significant main effect on number of entries into the open arms [$F(2,18) = 4.94$, $p < 0.05$]. Regardless of their exposure to acute stress, soman-challenged guinea pigs presented a lower number of entries into the open arms than saline-injected animals (Figure 4).

Two-way ANOVA revealed significant interactions between soman and stress on the total distance the animals traveled in the maze [$F(2,18) = 6.64$, $p < 0.01$] with no significant main effect of soman or stress on this parameter. Alone, soman or stress significantly shortened the total distance the animals traveled in the maze. In contrast, restraint-exposed animals that had been challenged with 0.8xLD50 soman exhibited the same level of locomotor activity as did non-restrained, saline-injected guinea pigs (Figure 4). Two-way ANOVA also showed significant interactions between soman and restraint stress [$F(2,18) = 5.72$, $p < 0.05$] with no main effects of either factor on number of closed arm entries (Table 1).

4. Discussion

The present study demonstrates that a single sub-lethal dose of soman or acute restraint stress triggers long-lasting anxiety-related behavior in guinea pigs, the best non-primate model of OP intoxication [15]. It also reveals that the magnitude of this behavioral effect is defined by significant interactions between the two factors, i.e. soman and acute stress.

When tested at 80–100 Lux in the open field with paper bedding-covered floor two months after a single exposure to a 2-h restraint stress, guinea pigs had lower exploratory activity than control animals. Distance traveled in the center of the field, a measure of anxiety, and total distance traveled in the field were shorter for restraint-stressed animals than for control animals. In addition, absence of movement in the open field, another measure of anxiety, was longer for restraint-stressed guinea pigs than for control animals. Under this experimental condition, soman (0.6 or 0.8xLD50)-challenged animals behaved as control animals. However, a significant interaction existed between the two factors (soman and acute restraint) and soman cancelled the effects of acute restraint.

Guinea pigs that were tested at 20–30 Lux in the open field with bare floor two months after the 2-h acute restraint stress also showed lower exploratory activity than control animals. In this environment, however, the anxiogenic effect of soman became evident. Animals that had been exposed to 0.6x or 0.8xLD50 soman alone exhibited lower exploratory activity than

control guinea pigs. These effects were long-lasting as they could still be detected three months after the initial exposures. A significant interaction occurred between soman and the acute restraint stress; the two factors together cancelled out each other's effects on the exploratory behavior of the animals in the open field. The finding that the center of the open field was less explored by guinea pigs challenged with a sub-lethal dose of soman or restraint stress than by control animals strongly indicates that either challenge triggered anxiogenic effects. However, the possibility exists that these challenges also affected the locomotor activity of the guinea pigs.

It is well known that both the activities of the hypothalamic-pituitary-adrenal (HPA) axis and of the mesostriatal and mesocorticolimbic dopaminergic pathways modulate anxiety-related behavior and locomotor activity of rodents in a given novel environment [25,26]. For instance, the anxiogenic effect of moving animals from their home cage into a novel environment is accompanied by activation of the HPA axis [27,28] and of catecholaminergic systems [29]. Likewise, in rats, the hyperlocomotor effect of amphetamine measured in the open field can be either dampened or potentiated by prefrontal cortical lesions, depending on the level of illumination of the field [30]. Under high-light conditions, which are more stressful for rodents than low-light conditions, the cortical lesions reduce amphetamine-induced hyperlocomotion [30]. The present findings demonstrate that the magnitude of the effects of soman and/or restraint stress on the exploratory activity of guinea pigs in the open field can be either blunted or facilitated depending on the background stress of the open field environment.

Significant interactions between a single exposure to sub-lethal doses of soman and acute restraint stress were also observed when the guinea pigs were tested three months later in the elevated plus maze. Animals that were exposed to either soman or acute restraint stress presented heightened anxiety, as they entered fewer times and spent less time in the open arms of the maze than did control animals. The findings that the animals challenged with soman or restraint stress travelled shorter distances in the maze and entered less in the closed arms than did control animals suggest that either challenge also decreased the locomotor activity of the guinea pigs. The finding of significant interactions between soman and stress and significant main effect of soman on the number of entries and time spent in the open arms suggest that pre-exposure to soman only partially reversed the anxiogenic effect of the acute stress. On the other hand, the finding of significant interactions between soman and stress with no significant main effect of either factor on number of entries in closed arms suggest that the two factors together cancelled out one another effects on locomotion.

As described in the Results session, the health status of one of the guinea pigs exposed to 0.8xLD50 soman was worsened by the acute restraint stress. This is in agreement with previous reports that acute stress can exacerbate the acute toxicity of soman [9,31]. Reduced AChE activity and increased ACh concentrations measured in the brain of rodents soon after acute restraint stress [8,32] could explain the stress-induced potentiation of the acute toxicity of soman. However, due to the low number of animals studied, one cannot rule out the possibility that the health status of the animal exposed to 0.8xLD50 would have worsened naturally with time after the exposure.

Restraint stress and AChE inhibitors such as soman, acting through different mechanisms, have many common effects in the brain, including: (i) activation of the immediate early gene c-fos, followed by increased expression of AChE and reduced expression of choline acetyltransferase – the enzyme that catalyzes the synthesis of ACh [33], (ii) activation of the HPA axis [34], and (iii) activation of catecholaminergic systems [35]. Thus, while activation of different signaling mechanisms by either restraint or soman can trigger a similar behavioral outcome, the simultaneous activation of these mechanisms by both factors acting in concert may cancel one another's effects. There are reports that the effects of stress on spontaneous and drug-induced behaviors vary depending on the duration and intensity of the stressors, number of exposures to the stressor, stress predictability, and strain of animals [36,37]. It is hitherto unknown how these parameters would modify the behavioral effects of sub-lethal doses of soman in guinea pigs.

In summary, long-lasting anxiety-related effects triggered by a single exposure to soman and/or stress were remarkably consistent in the different behavioral tests performed here. Therefore, the present study, though limited by the relative small sample size of the test groups, lays the groundwork for the exploration of the mechanistic basis underlying pathological conditions that are triggered by simultaneous exposures to stress and nerve agents.

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Figure 1. Body weight of guinea pigs subjected to different treatments

Graph presents weight gain of male guinea pigs that were subjected to one the following treatments: (i) saline (0.5 ml/kg, sc), (ii) saline (0.5 ml/kg, sc) followed 30 min later by 2-h restraint stress, (iii) 0.6xLD50 soman (16.8 µg/kg, sc), (iv) 0.8xLD50 soman (22.4 µg/kg, sc), (v) 0.6xLD50 soman followed 30 min later by 2-h restraint, or (vi) 0.8xLD50 soman followed 30 min later by 2-h restraint. On the day of the experiments (day 1), animals were weighted 1 h before the treatments. The weight gain of the animals was followed for three months. Data points and error bars represent mean and SEM, respectively, of results obtained from 3–5 animals/group.

Figure 2. Exploratory behavior of guinea pigs in the open field arena under different conditions

Graph shows the behavior of a set of control guinea pigs in the open field two months after their injection with saline (0.5 ml/kg, sc). On day 1, guinea pigs were tested for 10 min in the open field with bare floor. On day 2, the same animals were tested for 10 min in the open field with paper bedding-covered floor. Finally, on day 3, the guinea pigs were tested under the same conditions as on day 1. Light intensity was kept at 80–100 Lux throughout the experiments. Histogram and error bars represent mean and SEM, respectively, of results obtained from 4 animals/group.

Figure 3. Effects of a single exposure to soman and/or acute restraint on the exploratory behavior of guinea pigs tested in the open field

The same groups of animals described in Figure 1 were tested in the open field two and three months after their respective treatments. **A.** Results of tests performed two months post-treatments in the open field with floor covered with paper bedding. Light was kept at 80–100 Lux throughout the tests and behavior of each animal was recorded for 10 min. Two-way ANOVA revealed significant interactions between soman and restraint stress, with no main effect of either one on the parameters measured (see text). Post-hoc analysis with Bonoferroni's correction indicated that under this experimental condition restraint stress alone decreased the total distance the animals traveled in the field and increased the time they remained immobile (* $p < 0.05$, saline-restraint vs. saline), while soman alone had no

significant effect on any of the parameters measured. **B.** Results of tests of the same animals performed two and three months post-treatment in the open field with bare floor. Light intensity was kept at 20–30 Lux throughout the tests and behavior of each animal was recorded for 10 min. Total distance traveled, distance traveled in the center, and time immobile were measured. Three-way ANOVA revealed significant interactions between soman and restraint stress, with no main effect of either one on the parameters measured (see text). Post-hoc analysis with Bonferroni's correction indicated that under this experimental condition either restraint stress alone or soman alone decreased the total distance the animals traveled in the field and the distance they traveled in the center of the field, while increasing the time the animals remained immobile (* $p < 0.05$, vs. saline, † $p < 0.05$ vs. saline-restraint, ‡ $p < 0.05$ vs. respective non-restrained, soman-challenged group). In **A** and **B**, histogram and error bars represent mean and SEM, respectively, of results obtained from 3–5 animals.

Figure 4. Effects of a single exposure to soman and/or acute restraint on the behavior of guinea pigs in the elevated plus maze

Seven days after the last open field test, the guinea pigs were tested for 5 min in the elevated plus maze. Graphs show number of entries in the open arms, time spent in the open arms, and total distance traveled under each experimental condition. Two-way ANOVA revealed significant interactions and significant main effect of soman on the number of entries and time spent in the open arms, with soman reducing both parameters. Post-hoc analysis with Bonferroni's correction showed that soman alone or acute restraint alone significantly decreased the number of entries and time spent in the open arms (* $p < 0.05$, soman vs.

saline and saline-restraint vs. saline). Histogram and error bars represent mean and SEM, respectively, of results obtained from 3–5 animals.

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Table 1

Effects of a single exposure to soman and/or acute restraint stress on the number of entries of guinea pigs in the open and/or closed arms in the elevated plus maze.

	Number of Entries	
	Open arms	Closed arms
Saline	4.00 ± 0.82	7.75 ± 3.07
Soman 0.6xLD50	0.25 ± 0.25	1.00 ± 0.00
Soman 0.8xLD50	0.20 ± 0.20	1.6 ± 0.93
Saline-Restraint	0.25 ± 0.25	0.50 ± 0.29
Soman 0.6xLD50-Restraint	1.50 ± 0.87	3.50 ± 2.02
Soman 0.8xLD50-Restraint	0.67 ± 0.67	6.00 ± 3.21

Two-way ANOVA revealed significant interactions between soman and restraint stress, with no significant main effect of either factor on the number of entries in the closed arms (see text). Pairwise comparisons followed by Bonferroni's correction showed no significant difference among the test groups.