Animal models of PTSD: comparison of the neuroendocrine and behavioral sequelae of immobilization and a modified single prolonged stress procedure that includes immobilization

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Abstract

A single exposure to some stressors results in long-lasting consequences reminiscent of those found in post-traumatic stress disorder (PTSD), but results are very often controversial. Although there is no consensus regarding the best animal models of PTSD, the single prolonged stress (SPS) model, consisting of sequential exposure within the same day to various stressors (typically restraint, forced swim, and ether), has gained acceptance. However, results, particularly those related to the hypothalamic-pituitary-adrenal (HPA) axis, are inconsistent and there is no evidence that SPS is clearly distinct from models using a single severe stressor. In the present study, we compared in male rats the behavioral and neuroendocrine (HPA) consequences of exposure to immobilization on boards (IMO) with a SPS-like model (SPSi) in which IMO and isoflurane were substituted for restraint and ether, respectively. Both procedures caused a similar impact on food intake and body weight as well as on sensitization of the HPA response to a novel environment (hole-board) on the following day. Reduction of activity/exploration in the hole-board was also similar with both stressors, although the impact of sudden noise was higher in SPSi than IMO. Neither IMO nor SPSi significantly affected contextual fear conditioning acquisition, although a similar trend for impaired fear extinction was observed compared to controls. Exposure to additional stressors in the SPSi did not interfere with homotypic adaptation of the HPA axis to IMO. Thus, only modest neuroendocrine and behavioral differences were observed between IMO and SPSi and more studies comparing putative PTSD models are needed.

Keywords: PTSD, Immobilization, Modified Single Prolonged Stress, Fear Conditioning, Hypothalamic-Pituitary-Adrenal Axis, Homotypic Adaptation.

1. Introduction

Post-traumatic stress disorder (PTSD) in humans is a serious psychiatric disorder that has attracted considerable interest in the last decades. Exposure to traumatic experiences is considered the main cause of PTSD, a single experience being sufficient to produce PTSD in vulnerable subjects, although chronic exposure can exacerbate the consequences (Yehuda, 2002). The realization that PTSD could develop after a single exposure to a traumatic event prompted the study of the longlasting (days to weeks) behavioral consequences of a single exposure to various stressors in animals, with the aim of characterizing putative PTSD animal models (Yehuda and Antelman, 1993). Among them, acute exposure to inescapable electric shocks (IS), severe forms of restraint (REST), immobilization on boards (IMO), predators or predator odors (Armario et al., 2008; Deslauriers et al., 2018; Richter-Levin et al., 2019; Torok et al., 2019). However, the single prolonged stress model (SPS), originally described by Liberzon et al. (1997), is gaining acceptance as the preferred model of PTSD. SPS consists of sequential exposure to three stressors: 2 h REST, 30 min forced swim, and ether anesthesia (or more recently, other volatile anesthetics). In this model, the most extensively used behavioral tests are the elevated plus maze (EPM), the acoustic startle response (ASR), and fear conditioning, which mainly evaluate anxiety, arousal, and altered fear acquisition or extinction, respectively (Ferland-Beckham et al., 2021; Lisieski et al., 2018; Yamamoto et al., 2009).

Despite the good acceptance of the SPS model, results are far from being consistent. For instance, some studies reported enhanced fear acquisition (Imanaka et al., 2006; Iwamoto et al., 2007; Kohda et al., 2007; Takahashi et al., 2006), others impaired fear extinction (Yamamoto et al., 2008;), and some of them only detected impaired fear extinction recall (Ganon-Elazar and Akirav 2012; Knox et al., 2012a; Lin et al., 2016). These discrepancies could be due to differences in vulnerability between the different mouse or rat strains used, but certain modifications of the model are likely to contribute. One of these modifications is the substitution of ether anesthesia (prohibited in EU countries) for halothane or isoflurane (e.g. Ganon-Elazar and Akirav 2012; Harvey et al., 2003).

Recently, Sabban`s laboratory has used immobilization on boards (IMO) as an alternative to restraint, IMO being more severe than restraint (Campmany et al., 1996; García et al., 2000; Rabasa et al., 2015). They observed prominent and long-lasting changes in the hypothalamicpituitary-adrenal (HPA) axis and in anxiety-like behavior, supporting the validity of this modified SPS procedure (Laukova et al., 2014; Serova et al., 2013a, 2013b). The behavioral and particularly physiological consequences of IMO have been extensively studied for years to illustrate the consequences of exposure to severe stressors (e.g. Armario et al., 2004; Sabban and

Serova, 2007). Exposure to IMO has a strong physiological impact on the following days but less prominent long-term behavioral consequences (Armario et al., 2008). Nevertheless, during the first 10 days after a single IMO exposure, groups have reported impaired spatial memory in the Morris water maze (Andero et al., 2012) and enhanced ASR (Fuentes et al., 2014) in rats, and impaired fear extinction in mice (Andero et al., 2011). In addition to these behavioral consequences, we have repeatedly demonstrated that a single IMO exposure is enough to reduce the HPA response to a second exposure to IMO days or weeks later (Dal Zotto et al., 2004; Martí et al., 2001; Vallès et al., 2003). We have termed this phenomenon adaptation rather than habituation, considering that it did not follow the rules of habituation (Rabasa et al., 2015). These findings are clearly relevant regarding SPS as the integrity of the HPA axis is frequently assessed by exposing rats to restraint, one of the stressors included in the canonical SPS procedure (e.g., Kohda et al., 2007; Liberzon et al., 1997; Pooley et al., 2018). This might not be appropriate given that reduced response to the subsequent restraint might reflect adaptation after a single experience rather than altered functioning of the HPA axis (e.g., enhanced negative feedback).

The reasons why SPS is putatively better than other models are unclear from a theoretical perspective. Given the strong impact of IMO and the extensive use of the SPS procedure, it is appropriate to directly test whether a modified SPS procedure that includes IMO has more prominent effects than IMO alone and whether or not adaptation of the HPA axis to IMO might be affected by additional exposure to the other stressors of the SPSi procedure.

2. Methods

2.1. Animals and general procedures

Fifty-four male Sprague–Dawley rats obtained from the breeding centre of the *Universitat Autònoma de Barcelona* were used. The animals were housed in pairs and maintained under standard conditions of temperature (21 ± 1 ºC) and in a 12:12 h light/dark schedule (lights on at 07:00 h), with food and water available *ad libitum*. They were about 70 days old at the beginning of the experiments. The experimental protocol was approved by the Committee of Ethics of the *Universitat Autònoma de Barcelona* and by the *Generalitat de Catalunya*, and was carried out in accordance with the European Communities Council Directive 2010/63/EU and Spanish legislation. A maximal effort was made to minimize the number and suffering of animals.

2.2. Experimental design

The experimental treatments were always carried out in the morning. After arrival, rats were left undisturbed for 5 days, then handled four times, once every 2-3 days for approximately 2 min a day. After that, a first blood sample was taken by tail-nick under basal conditions to measure

resting levels of HPA hormones and habituate animals to the procedure. Tail-nick allows obtaining true basal levels of hormones (Belda et al., 2004; Vahl et al., 2005). Cage mates were sampled simultaneously (by two experimenters). Rat behavior was always videorecorded to be further analyzed. Each rat cage was randomly assigned to the different experimental groups:
control (n=18), IMO (n=18), and SPSi (N=18). See Fig. 1 for details of the procedure.

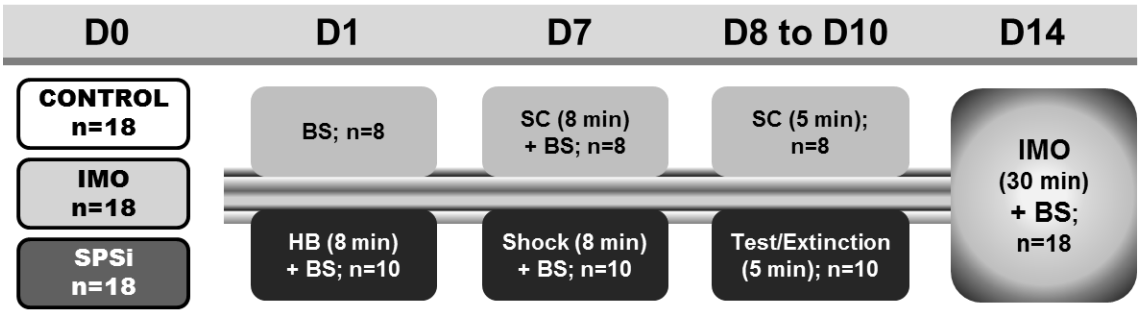

Figure 1: Design and timeline of experimental procedures. Rats were randomly assigned to three experimental groups: control (undisturbed), IMO or SPSi on day 0 (D0). Each group was subdivided into two subgroups: a first group (gray boxes) in which rats were not exposed to the hole-board (HB) on D1 and were exposed to the chamber but not shocked on D7, and a second group (black boxes) in which rats were exposed to the HB on D1 and shocks on D7. On D1, the rats from the first group were blood sampled (BS) to study the impact of the IMO and SPSi on basal levels of hypothalamic-pituitary-adrenal (HPA) hormones, whereas the rats from the second group were exposed to a HB for 8 min and then blood sampled. On D7, the rats from the first group were exposed to the shock chamber for 8 min without receiving shocks (SC), whereas those from the second group were shocked. On D7 both groups were blood sampled immediately after being taken from the chambers. From D8 to D10, all rats were exposed to the shock chamber to evaluate fear conditioning and extinction (no shocks). On D14, all rats were exposed to 30 min IMO stress and blood sampled.

The two rats from the same cage received identical treatment. Control rats remained undisturbed in the housing room. IMO rats were immobilized for 2 h by taping their four limbs to metal mounts attached to a board (Gagliano et al., 2008). Head movements were restricted with two plastic pieces (7 cm×6 cm) placed in each side of the head, and the body was subjected to the board by means of a piece of plastic cloth (10 cm wide) attached with *velcro* that surrounded all the trunk. Immediately after stress, IMO rats were returned to their home-cages and the housing room. In the SPSi procedure, immediately after the 2 h of IMO, rats were exposed individually to 20 min forced swim in a cylindrical tank (height 40 cm, diameter 19 cm) with 20 cm water at 25° C. After a 15 min resting period, rats were exposed individually to isoflurane anesthesia until loss of consciousness. Each stress procedure was done in a different room. On day 14, all rats were exposed to 30 min IMO and blood sampled immediately after the stressor (R0) and again 45 and 90 min later (R45 and R90, respectively).

2.3. Specific procedures

2.3.1. Control of food intake and body weight gain

Food intake and body weight are negatively affected by acute and chronic exposure to acute stressors, the impact being related to the intensity of stressors (Armario et al., 1990; Martí et al., 1994). In order to determine whether the impact of IMO and SPSi was different, we studied these parameters. The two measures were not always taken in parallel due to the possible interference of handling the animals to be weighed with the procedures carried out on particular days. For instance, on day 1, weighing animals prior to the HB exposure could interfere with behavior assessment and weighing after that could underestimate body weight due to the possible defecation rate associated with handling and HB exposure. For these reasons, the reference for body weight changes was taken the day before IMO or SPSi and the changes were studied 2, 3 and 7 days after the stressors. In contrast, changes in food intake were followed every day in the 3 post-stress days and on day 7. For food intake changes, the cage was considered as the experimental unit, and data was calculated by rat and day. In response to the fear conditioning procedure, the two variables were studied only in the next 24 h as we expected low responses if any.

2.3.2. Hole-board (HB) exposure

The HB consists of a rectangular white wooden box (62 × 53 × 28 cm) with a floor divided into 16 equal squares that contains four empty holes (diameter 4.5 cm) (File and Wardill, 1975a,b). This apparatus was chosen because it allows measuring both activity and exploration. Each animal was placed in the periphery of the apparatus facing the wall. The two animals of the same homecage were tested simultaneously in two independent HBs located in the same room. After an initial period of 5 min, animals were exposed to a sudden white noise (90 dB) for 30 sec, and their behavior was measured for an additional 2.5 min period. The noise was included to study whether the impact of a brief unexpected auditory stimulus could be modified by exposure to the stressors on the previous day. We measured the number of squares crossed (the two hindlimbs introduced simultaneously into a particular square), rearing episodes (both forelimbs off of the floor), and the number of head-dips (head introduced into the holes at least until eye-level). Time spent with the body and head totally immobile was considered freezing. The apparatus was carefully cleaned between animals with soapy water.

2.3.3. Context fear conditioning (CFC)

Seven days after IMO or SPSi rats were exposed to CFC in a rectangular Plexiglas chamber (57 x 41 x 70 cm) with an electrified grid (0.4 cm rods, 1.5 cm apart) and an anterior transparent wall. After a 3 min pre-shock exposure, rats to be conditioned received 3 foot-shocks (3 sec, 0.5 mA,

1 min interval between shocks) and remained in the apparatus for another 3 min. The nonconditioned rats were merely exposed for 8 min to another chamber placed in a different room. On the three following days, they were exposed again for 5 min to the apparatus without shocks to test fear conditioning and extinction. Shock chambers were always cleaned between rats with 5% ethanol during conditioning and tests, which served as an additional contextual cue. Time spent freezing (total body and head immobility in a rigid body position) was manually measured. Total horizontal activity was measured using video-tracking (Smart video tracking, Panlab/Harvard Instruments).

2.3.4. Blood analysis

Plasma ACTH and corticosterone levels were determined by well-established double-antibody radioimmunoassay (RIA) procedures, as described previously (Muñoz-Abellán et al., 2011). All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 5.1% for ACTH and 7.6% for corticosterone. The sensitivity of the assays was 25 pg/ml for ACTH and 2 ng/ml for corticosterone.

2.4. Statistics

Data were analyzed by the SPSS-IBM software (version 24). Generalized linear models (GzLM) were used when only between-subjects factors were included or general estimated equations (GEE) when within-subjects factors were also considered (McCulloch and Searle, 2001; Hardin and Hilbe, 2003). These models have the advantage of not requiring normality or homogeneity of variances, although hormonal data were log. transformed to improved homogeneity. Results were considered significant when $p< 0.05$, although marginally significant differences (p between 0.1 and 0.05) were also indicated regarding interaction between factors.

3. Results

3.1. Changes in food intake and body weight

Food intake and body weight changes were analyzed by GEE, with prior STRESS (control, IMO, SPSi) as the between-subjects factor and DAY as the within-subjects factor (Figure 2). The GEE analysis of food intake showed effects of prior STRESS ($\chi^2_{(2)}= 15.0$, $p= 0.001$), DAY ($\chi^2_{(3)}= 140.8$, $p< 0.001$) and the interaction STRESS x DAY ($\chi^2_{(6)}= 69.7$, $p< 0.001$). Decomposition of the interaction showed reduced food intake in IMO and SPSi versus controls from days 1 to 3 but not on day 7 (see Figure 2 for detailed p values). IMO resulted in lower food intake than SPSi on day 1 ($p= 0.004$). The GEE analysis of changes in body weight gain (Figure 2) showed significant effects of STRESS ($\chi^2_{(2)}= 84.4$, $p< 0.001$), and DAY ($\chi^2_{(2)}= 1301.4$, $p< 0.001$) but not of the interaction STRESS x DAY, with no differences between IMO and SPSi.

Food intake and body weight changes in the 24 h following fear conditioning were analyzed by GzLM, with prior STRESS and SHOCK exposure as the between-subjects factors. No significant effects of either STRESS or SHOCK on food intake were found, whereas body weight changes showed no significant effect of STRESS, but a significant effect of SHOCK ($\chi^2_{(1)}$= 4.9, p= 0.027), which reduced body weight gain (data not shown).

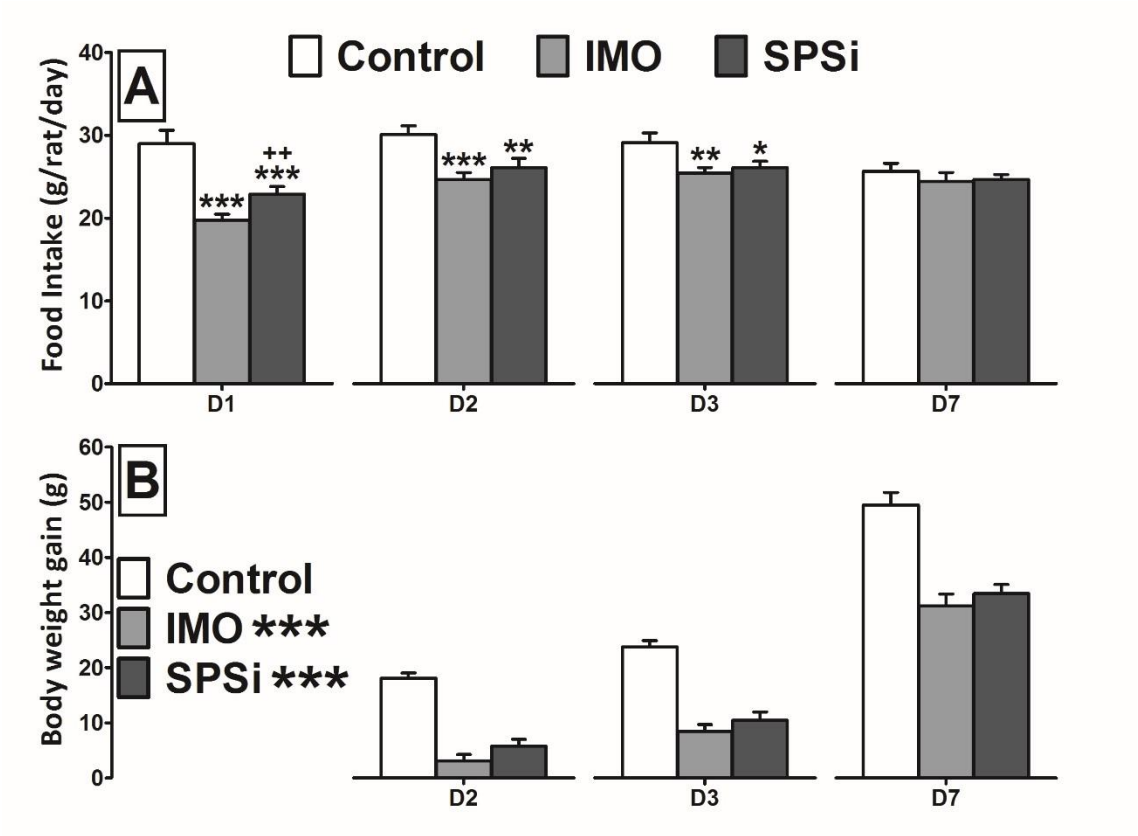

Figure 2: Impact of IMO and SPSi on food intake (A) and body weight (B). Means and SEM are represented (n=9 for food intake and n= 18 for body weight gain). Food intake was measured daily from day (D) 1 to D3 post-stress and again on D7. *** p< 0.001 versus controls within the same day; ++ p< 0.01 vs IMO group within the same day. Cumulative body weight gain was evaluated on D2, D3, and D7 post-stress with respect to the baseline before stress exposure (it was not measured on D1 to avoid interference with behavioral tests). *** p< 0.001 vs. controls over the entire period measured.

3.2. Response to the HB

The behavior was expressed as episodes in 30 sec periods in order to better compare behavior before, during and after noise exposure (Figure 3). The GEE analysis included one betweensubjects factor (prior STRESS: control, IMO, and SPSi) and one within-subjects factor (TIME: pre-noise, noise, post-noise). Regarding ambulation, significant effects of STRESS ($\chi^2_{(2)}$= 13.7, p< 0.001), TIME ($\chi^2_{(2)}$= 83.0, p< 0.001) and the interaction STRESS x TIME ($\chi^2_{(4)}$= 16.1, p< 0.003) were found. Decomposition of the interaction showed lower ambulation in the IMO and SPSi groups than in controls during the pre-noise period (p< 0.001 in both cases), lower

ambulation in the SPSi than control rats during noise (p= 0.009), and significant decreases in control and SPSi groups, but not in the IMO group, in the post-noise versus the pre-noise period (p< 0.001 in the two cases). Regarding rearing, a marginal effect of STRESS ($\chi^2_{(2)}$= 5.6, p= 0.06) and a significant effect of TIME ($\chi^2_{(2)}$= 135.7, p< 0.001) were found, with no interaction; in the three groups, exposure to noise resulted in a marked decrease in rearing during the post-noise versus the pre-noise period (always p< 0.001). Results regarding head-dipping showed a marginally significant effect of STRESS ($\chi^2_{(2)}$= 4.9, p= 0.09), TIME ($\chi^2_{(2)}$= 21.1, p< 0.001), and the interaction STRESS x TIME ($\chi^2_{(4)}$= 14.1, p=0.007). Decomposition of the interaction showed lower ambulation in IMO and SPSi than in control rats during the pre-noise period (p= 0.005, p= 0.025, respectively). Comparison of the post-noise versus the pre-noise period showed a significant decrease in controls (p< 0.001), a marginally significant decrease in the SPSi (p=0.076), and no decrease in the IMO group.

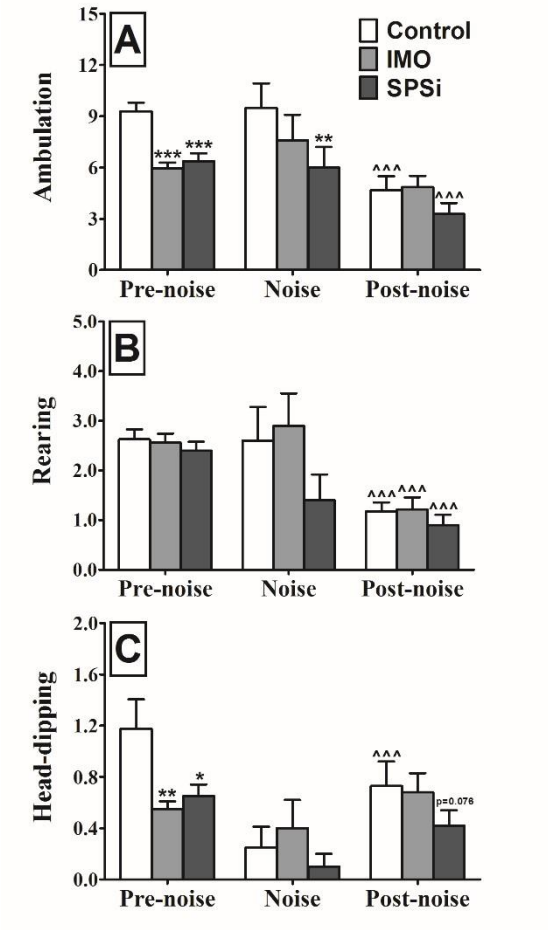

Figure 3: Impact of IMO and SPSi on behavior in the hole-board (HB) measured on the day after stress (D1): ambulation (A), rearing (B), and head-dipping (C). Means and SEM (n=10 except for controls, n=8) are represented. Behavior was evaluated during the first 5 min period (prenoise), during exposure to noise for 30 s, and 2.5 min after noise (post-noise), and represented in events per 30 s to allow comparison of the different periods. *** p< 0.001, ** p< 0.01, * p< 0.05 versus controls within the same period. ∧ ∧ ∧ p< 0.001 versus the same group in the prenoise period (a marginally significant effect was found in the SPSi group.

The basal activity of the HPA axis and its response to the HB (a heterotypic stressor) was studied the day after stress exposure (Figure 4), with prior stress (STRESS) and HB exposure as the between-subjects factors. The ACTH results revealed effects of STRESS ($\chi^2_{(2)}$= 32.5, p< 0.001)

and HB ($\chi^2_{(1)}$= 148.2, p< 0.001), but not of the interaction. Further comparisons showed higher levels of ACTH, regardless of HB exposure, in both IMO and SPSi than in control groups (p < 0.001 in the two cases), with no differences between IMO and SPSi. Similar results were obtained with corticosterone: effects of STRESS ($\chi^2_{(2)}$= 17.5, p< 0.001) and HB ($\chi^2_{(1)}$= 121.69, p< 0.001), but no interaction, and higher levels in both IMO and SPSi than controls (p< 0.001 in the two cases). These results demonstrate that prior exposure to the two stressors increased both resting and post-HB levels of the two hormones.

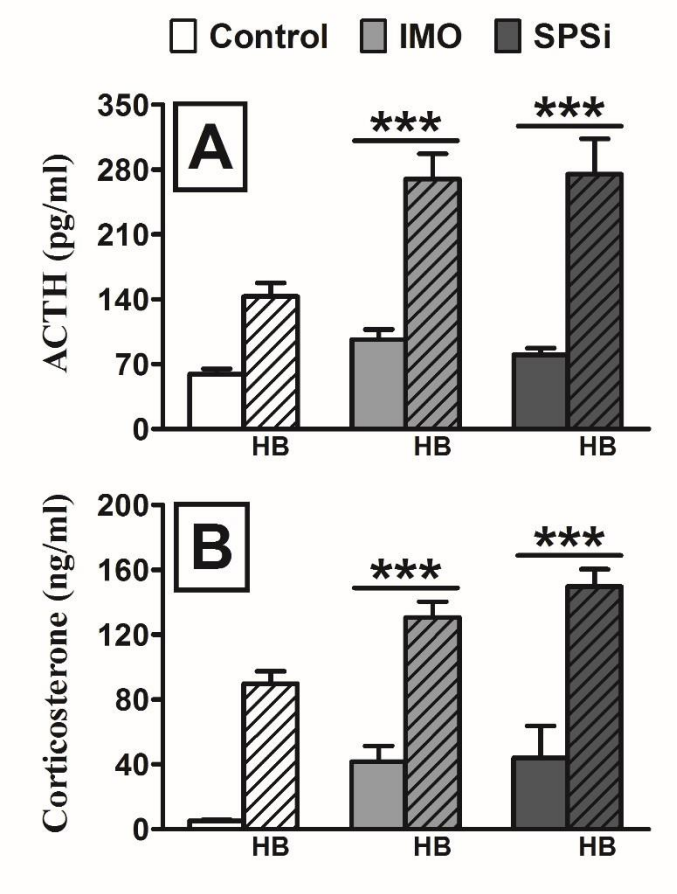

Figure 4: Impact of IMO and SPSi on resting levels of ACTH (A) and corticosterone (B) and their response to the 8 min hole-board (HB) exposure on the day after the stressors. Means and SEM (n=8 for resting levels and n=10 for HB exposure) are represented. *** p< 0.001 vs. controls, regardless of acute condition (basal or HB).

3.3. Fear conditioning and extinction

The behavior of non-fear conditioned rats in the shock chamber was measured with no differences between stress groups and over the days. Freezing was basically absent (average values between 2 and 11 seconds in a 5 min period), and distance moved was, on average, around 1100-1200 cm in all groups and sessions (Table 1). The statistical analysis was done only in fear-conditioned rats. During training, pre-shock freezing was very low in all groups and greatly increased in the post-shock period, with no differences between the three groups (Figure 5A). An opposite pattern was found regarding activity (Figure 5B), again with no group differences.

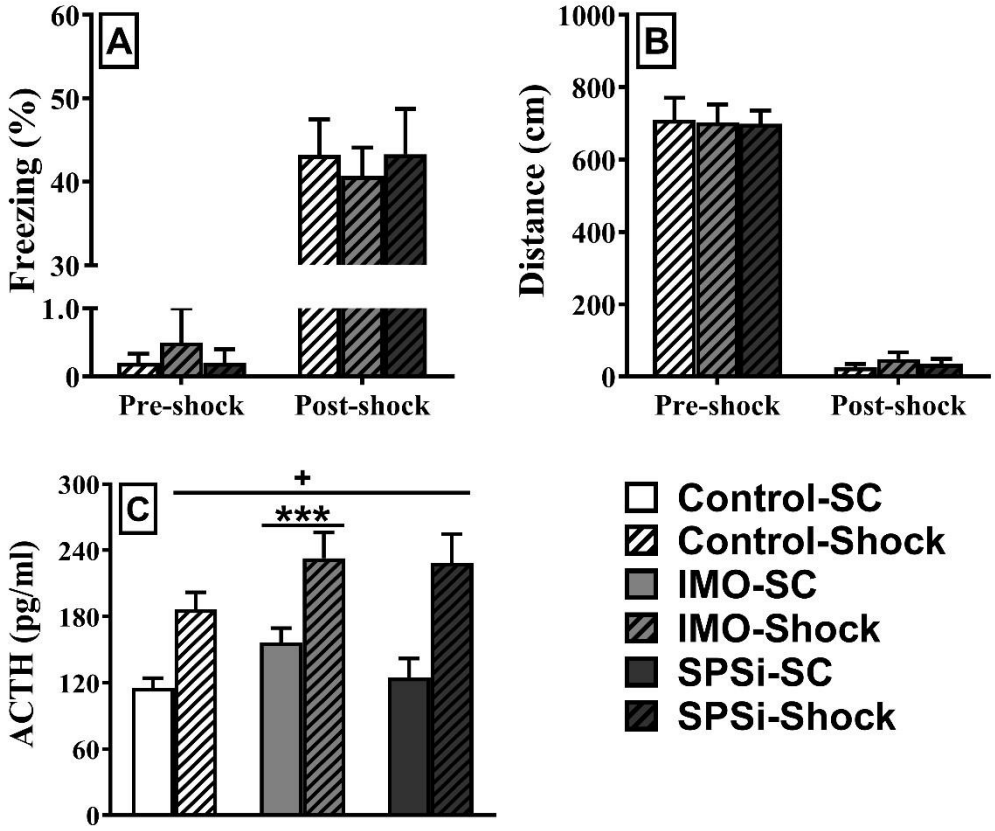

Figure 5: Impact of IMO and SPSi on fear conditioning training. Means and SEM are represented. Panels A and B show the percent time spent freezing and distance moved before and after shock exposure during the conditioning session in the three shocked groups (n=10): no group differences were found. Panel C shows ACTH levels immediately after exposure to the shock chamber (SC) without shocks (n=8) for 6 min or after receiving the single shock (n=10). ACTH levels increased in all groups after the shock (overall effect + $p < 0.05$ versus the SC groups), and values were greater in IMO (but not SPSi) versus control rats, regardless of shock (*** $p < 0.001$).

The ACTH levels after exposure to the shock context or to shock during conditioning training were assessed with STRESS and SHOCK as the main factors (Figure 5C and 5D). The GLzM analysis revealed significant effects of STRESS ($\chi^2_{(2)} = 7.0$, $p < 0.05$) and SHOCK ($\chi^2_{(1)} = 37.7$, $p < 0.001$) but not the interaction. Further comparisons revealed a significantly greater response in the IMO, but not in the SPSi group, with respect to the controls, although the pattern was similar in IMO and SPSi groups.

Behavioral response to CFC testing and extinction was analyzed by GEE with STRESS and DAY as the main factors. Freezing significantly decreased over the days (DAY: $\chi^2_{(2)} = 45.1$, $p < 0.001$), whereas activity increased ($\chi^2_{(2)} = 104.7$, $p < 0.001$). However, no effect of STRESS or the interaction DAY x STRESS was found. As the IMO and SPSi groups followed the same pattern during extinction, both groups were pooled to be compared with controls (Figure 6). The analysis of freezing showed significant effects of STRESS ($\chi^2_{(1)} = 4.4$, $p = 0.036$) and DAY ($\chi^2_{(2)} = 50.6$, $p <$

0.001), but no interaction, indicating enhanced levels of freezing in the previously stressed versus control rats, regardless of day. Although visual inspection of the data suggests a greater impact of prior stress on extinction, the lack of interaction precluded comparisons within each specific day. The analysis of distance showed a marginally significant effect of STRESS ($\chi^2_{(2)}$= 3.5, p= 0.06) and significant effects of DAY ($\chi^2_{(2)}$= 127.9, p< 0.001) and the interaction STRESS x DAY ($\chi^2_{(2)}$= 8.3, p= 0.016). Decomposition of the interaction revealed marginally significant reduced activity in STRESS versus control groups on the 2nd extinction day (p= 0.053) and significantly reduced levels on the 3rd extinction day (p= 0.04).

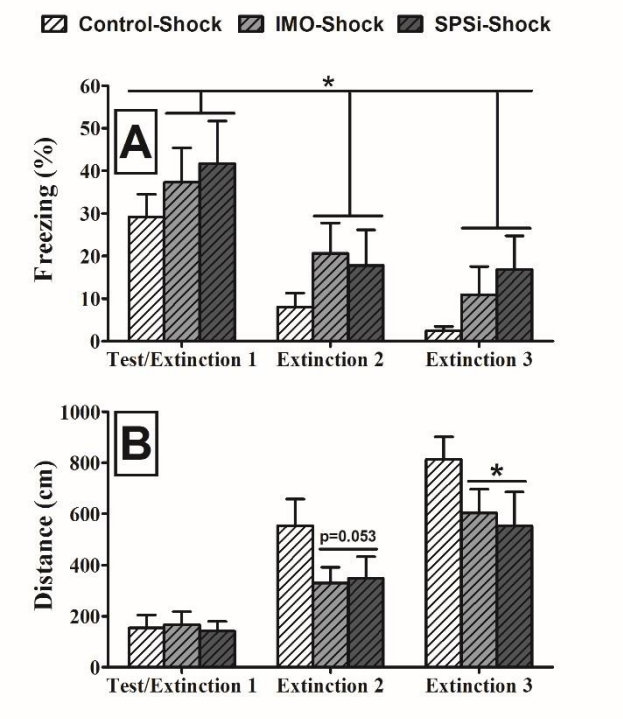

Figure 6: Effect of prior IMO or SPSi on contextual fear conditioning memory and extinction. Means and SEM (n=10) of the percent time spent freezing (A) and distance moved (B) in fear-conditioned rats are represented. Non-fear-conditioned rats showed almost no freezing, and distance moved did not differ between the three stress groups and days (around 1200, not shown). The overall pattern of fear-conditioned rats demonstrated extinction of fear over days with progressive decreases in freezing and increases in activity (significance not shown). When IMO and SPSi rats were pooled, the analysis of freezing revealed significant overall group differences across the days, whereas the analysis of distance moved showed impaired extinction in previously stressed (IMO or SPSi) animals, * p< 0.05 vs. controls (regardless of session).

Table 1. Distance moved in the shock chamber in non-shocked groups.

Group (n)	Test/Extinction 1	Extinction 2	Extinction 3
Control (7)	1215 ± 133	1018 ± 107	1139 ± 127
IMO (8)	1133 ± 121	947 ± 177	1166 ± 158
SPSi (8)	1208 ± 112	1129 ± 124	1222 ± 126

Data are expressed as Mean ± SEM. No group differences were observed.

3.4. Adaptation to the homotypic stressor

The ACTH and corticosterone levels in response to 30 min IMO are depicted in Figure 7. All rats were exposed to 30 min IMO two weeks after stress exposure and sampled just after IMO (IMO30) and 45 and 90 min after IMO (R45 and R90). The analysis showed significant effects of STRESS ($\chi^2_{(2)}$= 9.5, p< 0.01), TIME ($\chi^2_{(2)}$= 2091.2, p< 0.001), and the interaction STRESS x TIME ($\chi^2_{(4)}$= 23.2, p< 0.001), but no effect of SHOCK (fear conditioning). Decomposition of the interaction showed similar ACTH levels just after IMO but reduced levels at the two post-IMO periods (R45 and R90) in both IMO and SPSi pre-exposed groups (p< 0.001 except for R90 in the IMO group, p< 0.01). The analysis of corticosterone showed significant effects of STRESS ($\chi^2_{(2)}$=32.3, p< 0.01), TIME ($\chi^2_{(2)}$= 450.6, p< 0.001), and the interaction STRESS x TIME ($\chi^2_{(4)}$= 53.0, p< 0.001), but no effect of SHOCK (fear conditioning). Decomposition of the interaction showed higher corticosterone levels in the SPSi group versus controls just after termination of IMO (p< 0001) but lower levels at R45 (p=0.029) and R90 (p< 0.001). In the IMO group, lower levels than in controls were found at the two post-IMO periods (p< 0.001). Only at R90 were differences found between IMO and SPSi groups, with higher levels in the SPSi group (p= 0.013).

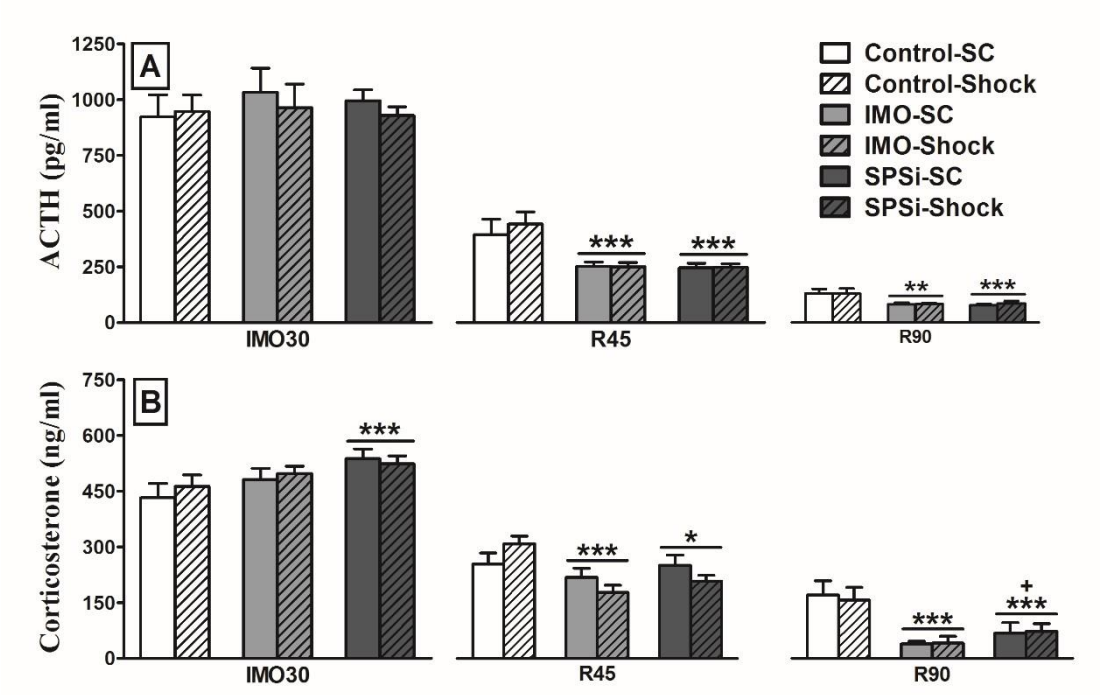

Figure 7: Effect of prior IMO or SPSi exposure on ACTH (A) and corticosterone (B) response to an acute IMO 14 days after initial exposure to the stressors. Means and SEM (n=8-10) are represented. All animals were exposed to 30 min IMO with blood sampled immediately after the stressor (IMO30) and 45 and 90 min after stress termination (R45 and R90, respectively). Prior IMO experience (alone or within the SPSi procedure) resulted in faster recovery of prestress ACTH and corticosterone levels when all rats were re-exposed to IMO. SPSi rats also showed higher corticosterone levels than controls just after IMO and higher levels than IMO rats at R90. No effect of prior exposure to shock was observed; *** p < 0.001, ** p< 0.01, * p< 0.05 vs. controls, and + p< 0.05 vs. IMO, within the same sampling time.

4. Discussion

The present experiment is the first one comparing IMO and a modified SPS procedure that also included IMO. Only relatively modest differences in physiological and behavioral effects were observed between the two procedures, although not always in the same direction, suggesting a minor contribution of additional forced swim and isoflurane anesthesia exposure.

As expected from severe stressors (Márquez et al., 2002; Martí et al., 1994; Vallès et al., 2000), IMO and SPSi significantly reduced food intake and body weight gain for several days, with a progressive normalization. However, somewhat surprisingly, their impact on food intake was initially greater after IMO than after SPSi. Whether anesthesia can affect the impact of IMO is unknown, but the results conclusively demonstrated no additional impact of exposure to forced swim and isoflurane anesthesia.

The day after exposure to IMO or SPSi, the two stressed groups showed a similar reduction of ambulation and number of head-dips in the HB compared to controls, whereas rearing activity was not altered. The negative impact of severe stressors on activity in novel environments is in accordance with prior studies (Belda et al., 2016; Kennett et al., 1985; Lehnert et al., 1984). Group differences were also found after a brief loud noise was superimposed. In the post-noise period, all groups showed reduced activity with respect to the pre-noise period, but the impact in IMO and SPSi groups was relatively lower perhaps due to the pre-noise hypoactivity in these groups. A greatest sensitivity appears to exist in SPSi versus IMO. The actual factors underlying the negative impact of severe stressors on activity/exploration in novel environments over the next 24 h are not well-known. Enhanced anxiety might contribute, but we did not observe specific differences in central versus peripheral exploration or in freezing.

Both IMO and SPSi significantly increased to the same degree basal levels of ACTH and corticosterone on the day after the stressors, in full agreement with previous results using IS, IMO, or SPS (Belda et al., 2020; Fleshner et al., 1995; Ganon-Elazar and Akirav, 2012; Kohda et al., 2007; Martí et al., 1996; Ottenweller et al., 1994). Importantly, sensitization of the ACTH and corticosterone responses to the HB was found after both IMO and SPSi, supporting that they are severe stressors (Belda et al., 2008; 2012; 2016; Johnson et al., 2002; O'Connor et al., 2003e). When rats were exposed to the CFC training 7 days after IMO or SPSi, a modest but still significant ACTH sensitization was observed in the IMO group. The same pattern was found in the SPSi group, although it did not reach significance. It thus appears that a residual sensitization of the HPA axis persisted 14 days after IMO and SPSi exposure, supporting the durability of the phenomenon (Belda et al., 2015).

In the period of habituation to the shock chamber, activity was similar in all groups and markedly decreased immediately after shocks. Reduced activity and freezing reflecting fear did not differ among the groups during testing for fear conditioning one day later, but in the next exposures to the context, when evidence for extinction emerged, both IMO and SPSi showed a similar tendency for impaired extinction. Although differences with respect to controls did not reach significance, when the two groups were considered together, overall higher levels of CFC were observed in previously stressed versus control rats, suggestive of modestly impaired extinction, which is in accordance with an important number of previous studies using IMO in mice (Andero et al., 2011) or the SPS procedure in rats and mice (Ganon-Elazar and Akirav 2012; Knox et al., 2012a; Lin et al., 2016; Yamada et al., 2011; Yamamoto et al., 2008).

Regarding the possible interference of the additional stressors used in our SPS procedure in the homotypic adaptation of the HPA axis to IMO, we observed, as expected from our previous results, that a single exposure to IMO was enough to reduce the HPA response to a second exposure, the effect being evident during the post-IMO recovery period (Dal-Zotto et al., 2004; Martí et al., 2001; Rabasa et al., 2015). Importantly, no interference was found by the other additional stressors of the SPSi. This is in line with previous results showing that administration of lithium during exposure to IMO did not interfere with adaptation despite evidence for lithiuminduced malaise (Sanchis-Ollé et al., 2017). These results support that homotypic adaptation is an extremely robust phenomenon and raise the question of independence versus interaction regarding how the brain processes different stressors when presented simultaneously or with a short delay between them. The present results are also compatible with some studies demonstrating anterograde but not retrograde interference of isoflurane with memory of shockinduced fear (Bunting et al., 2016; Dutton et al., 2002).

The fact that prior IMO experience reduced the HPA response to the same stressor strongly recommends ruling out those stressors included in the SPS procedure to study the integrity of negative glucocorticoid feedback (Kohda et al., 2007; Liberzon et al., 1997; Pooley et al., 2018). This is particularly relevant when relying only on corticosterone, which reflects ACTH release with important limitations (see Armario, 2006). Nevertheless, other authors have demonstrated SPS-induced enhanced negative glucocorticoid feedback in response to novel (heterotypic) stressors (Ganon-Elazar and Akirav, 2012), supporting the possibility that the SPS exposure actually enhanced negative feedback.

The present data do not support the hypothesis that our modified SPSi procedure has some particular properties that makes it a more appropriate animal model of PTSD than IMO alone. It has been argued that ether exposure is very relevant (Knox et al. 2012b). However, there are no

obvious theoretical reasons for this assumption other than the fact that ether is probably a more severe systemic stressor than isoflurane (Arnold and Langhans, 2010). Importantly, other authors have used isoflurane and still observed changes reminiscent of those observed in PTSD (GanonElazar and Akirav, 2012). Based on other results, substituting restraint with IMO maintains relevant characteristics of the model while perhaps increasing its impact on the HPA axis (Serova et al., 2013b; 2014; 2019). In parallel with a more exhaustive exploration of the contribution of the different stressors in SPS or SPS-modified models, discrepancies in the literature are likely to be in part explained by possible differences in the susceptibility of rat or mouse strains used in the different laboratories (Armario and Nadal, 2013). To our knowledge, strain differences in the impact of SPS have not been studied. Although the present study included only male rats, we have not observed evidence for enhanced sensitivity of females versus males in the consequences of a single IMO exposure (Gagliano et al., 2014).

Considering the well-established fact that early life stress (ELS) enhanced the susceptibility to PTSD after adult exposure to traumatic stressors (Breslau et al., 1995; Hodes and Epperson, 2019; Ochi and Dwivedi, 2022), an additional possibility to improve the translational value of acute severe stressors during adulthood as putative PTSD animal models is to combine them with ELS. Unfortunately, experimental results are still controversial, and sex differences add complexity to the issue (e.g., Chaby et al., 2020; Cheng et al., 2019; Fuentes et al., 2018; Knox et al., 2021; Mancini et al., 2021; Tsoory et al., 2007). A more precise framework about the nature and timing of stress exposure and the origin of sex differences are needed in preclinical and clinical studies (Hodes and Epperson, 2019).

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