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ORIGINAL ARTICLE

Genes, Brain and Behavior

Neuropeptide-S-receptor deficiency affects sex-specific modulation of safety learning by pre-exposure to electric stimuli

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Abstract

Neuropeptide S (NPS) is a neuropeptide involved in the regulation of fear. Because safety learning is impaired in patients suffering from anxiety-related psychiatric disorders, and polymorphisms of the human neuropeptide S receptor (NPSR) gene have also been associated with anxiety disorders, we wanted to investigate whether NPSR-deficiency interferes with safety learning, and how prior stress would affect this type of learning. We first investigated the effect of pre-exposure to two different types of stressors (electric stimuli or immobilization) on safety learning in female and male *C57Bl/6 mice*, and found that while stress induced by electric stimuli enhanced safety learning in males, there were no differences in safety learning following immobilization stress. To further investigate the role of the NPS system in stress-induced modulation of safety learning, we exposed NPSR-deficient mice to stress induced by electric stimuli 10 days before safety learning. In nonstressed male mice, NPSR-deficiency enhanced safety learning. As in male *C57Bl/6 mice*, pre-exposure to electric stimuli increased safety learning in male NPSR +/+ mice. This pre-exposure effect was blocked in NPSR-deficient male mice showing impaired, but still intact, safety learning in comparison to their NPSR +/+ and NPSR +/− littermates. There was neither a pre-exposure nor a genotype effect in female mice. Our findings provide evidence that pre-exposure to stress induced by electric stimuli enhances safety learning in male mice, and that NPSR-deficiency prevents the beneficial effect of stress exposure on safety learning. We propose an inverted U-shape relationship between stress and safety learning.

KEYWORDS

anxiety, behavioral therapy, conditioned safety, fear conditioning, immobilization, neuropeptide S, stress

1 | INTRODUCTION

Neuropeptide S (NPS) is a regulatory neuropeptide that is highly conserved among vertebrates.[1] Although the expression of NPS seems to be restricted to only few glutamatergic neurons that are primarily localized in the pericoerulear area and the Kölliker-Fuse nucleus, the NPS-Receptor (NPSR) is widely distributed across the mouse brain and highly abundant in the thalamic and hypothalamic regions, the

basolateral amygdala, the subiculum, and many cortical areas.[2] A number of studies addressed the behavioral consequences of NPS administration in mice with the general consensus that NPS produces anxiolytic-like effects.[3-6] Studies investigating the effects of genetic or pharmacological manipulation of the NPS system have further demonstrated that NPS plays an important role in the regulation of fear behavior. For instance, it has been shown that intracerebral NPS injections lead to a reduction of conditioned fear and even rescue stress-induced fear extinction deficits.[7-10]

Similar, but distinct from fear extinction learning, is safety learning. Here, a safety cue predicts the absence of an aversive event, thereby inhibiting fear responses to a fear-associated context or cue.[11,12] Several lines of research indicate that safety learning is impaired in patients suffering from anxiety disorders, such as panic disorder or post-traumatic stress disorder.[13-16] Interestingly, genetic studies in humans have found that several single-nucleotide polymorphisms and splice variants of the human NPSR gene have been associated with higher incidences of anxiety disorders.[17-21] Nevertheless, up until now, no human or rodent study investigated the role of the NPS system in safety learning.

The aim of the present study was to investigate whether NPSR-deficiency interferes with safety learning, and how prior stress, as most often seen in patients suffering from anxiety disorders, affects this type of learning. We first tested the effects of pre-exposure to two different types of stressors on safety learning in female and male *C57Bl/6 mice*. In the first experiment, the effect of immobilization stress on safety learning was tested. In a second experiment, mice were exposed to electric stimuli and submitted to safety learning. In the third experiment, we tested the role of the NPS system in stress-induced modulation of safety learning. For this, female and male NPSR-deficient mice were exposed to electric stimuli, and then submitted to safety learning. Because the NPS system has been shown to modulate the effects of stress on emotional learning,[10] we hypothesized that NPSR-deficiency will affect the modulation of safety learning by prior stress.

2 | MATERIAL AND METHODS

2.1 | Animals and housing conditions

Experiment 1 was conducted at the Institute of Neurosciences, Autonomous University of Barcelona, Spain. Experimental subjects were adult male and naturally cycling female C57BL/6J mice (♂: n = 16, ♀: n = 16), aged 8 weeks and obtained from Charles River Spain. Mice were group-housed by sex (four animals/cage) in transparent Tecniplast 1145 T cages (435 cm^2) with wood chip bedding and nesting material. The animals had free access to standard chow (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and filtered tap water, with a fixed 12:12 hour light/dark photoperiod (lights on at 08:00 hour) in a temperature- (21 ± 2°C) and humidity-controlled (50 ± 5%) room.

Experiments 2 and 3 were conducted at the Institute of Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Germany. Experimental subjects were adult 8 to 12 weeks old male and naturally cycling female C57BL/6J mice (♂: n = 45, ♀: n = 47; experiment 1), as well as homozygous neuropeptide S receptor knockout mice (NPSR −/−; ♂: n = 23, ♀: n = 24), and their heterozygous (NPSR +/−; ♂: n = 21, ♀: n = 23) and wild-type (NPSR +/+; ♂: n = 20, ♀: n = 24) littermates (experiment 3). The NPSR mice[22] were backcrossed to C57BL/6J mice (Origin: Charles River, Germany) for more than 20 generations, with our local NPSR mouse breeding stock existing for 7 years (ca. 10-12 generations). All mice were bred in the institutes' animal facility and group-housed by sex (4-6 littermates/cage) in transparent Makrolon Type III cages (840 cm^2) with wood chip bedding, nesting material and cage enrichment. The animals had free access to standard chow (Ssniff R/M-H, V1534-0, Germany) and tap water, with a fixed 12:12 hour light/dark photoperiod (lights on at 06:00 hour) in a temperature- (22 ± 2°C) and humidity-controlled (50 ± 5%) room.

All experimental procedures were approved by the local authorities (Committee of Ethics of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, Spain: #9626; Landesverwaltungsamt Sachsen-Anhalt: Az. 42 502-2-1309 Uni MD), and conducted in agreement with international guidelines and regulations for animal experiments (2010/63/EU).

2.2 | Behavioral testing

2.2.1 | Stress by immobilization (experiment 1)

The immobilization procedure was conducted in an unfamiliar environment, separated from the housing and behavioral test rooms. Mice were immobilized for 2 hours by restraining each of their four limbs to metal arms attached to a wooden board.[23] Control animals were handled for 2 minutes by letting the animals walk on the experimenters' hands. All cage mates received the same treatment. Following treatment, mice were returned to their homecage where they remained undisturbed until safety learning 10 days later.

2.2.2 | Stress by electric foot shocks (experiment 2 and 3)

Exposure to the electric stimuli was conducted in an unfamiliar environment, separated from the housing and behavioral test rooms. Electric stimuli were administered using the foot shock generators of a startle system with eight chambers (SR-LAB, San Diego Instruments, USA). The mice were put into transparent animal enclosures (4 cm × 10 cm; 10 lx; no background noise; no odor). After an acclimation time of 5 minutes, five scrambled electric stimuli (0.7 mA, 1 second) were administered with an inter-stimulus interval of 30 seconds via a floor grid (1.5 mm in diameter with a distance 5 mm between bars). The delivery of electric stimuli was controlled by the SR-LAB software (SR Lab Pt. #6500-0087-B, 2002). Control animals underwent the identical procedure without receiving electric stimuli. All cage mates received the same treatment. Following treatment, mice

were returned to their homecage where they remained undisturbed until safety learning 10 days later.

2.2.3 | Safety learning

2.2.4 | Conditioning setup (experiment 1)

For the safety learning procedure in Experiment 1, we used a computerized *StartFear Combined* system (Panlab-Harvard, Barcelona, Spain), consisting of a black methacrylate box with a transparent front door (25 cm × 25 cm × 25 cm). The box was located in a sound-attenuating chamber (67 cm × 53 cm × 55 cm) equipped with loud-speakers for acoustic stimuli, a light source (continuous background illumination of ~10 lx) and a ventilation fan, producing a background noise of approximately 55 dB. The floor of the boxes consisted of removable stainless steel grids (3 mm in diameter with a distance 10 mm between bars) which were connected to a shock unit and able to deliver electric stimuli. Delivery of all stimuli was controlled by the Panlab Software (Freezing 1.3.04, Panlab-Harvard, Barcelona, Spain). Movements of the animals were detected through a highly motion-sensitive transducer system.

2.2.5 | Conditioning setup (experiment 2 and 3)

For the safety learning procedure in experiment 2 and 3, we used a computerized fear-conditioning system (TSE Systems, Bad Homburg, Germany), consisting of four transparent Perspex box (46 cm × 46 cm × 32 cm) that were surrounded by infrared animal detection sensor frames. The boxes were located in sound-attenuating chambers equipped with loudspeakers for acoustic stimuli, a light source (continuous background illumination of ~20 lx) and ventilation fans, producing a background noise of approximately 55 dB SPL. The floors of the boxes consisted of removable stainless steel grids (bars: 4 mm in diameter with a distance of 9 mm between the bars) that were connected to a shock unit and able to deliver electric stimuli. Delivery of all stimuli was controlled by the TSE FC Software (V9.10). To ensure that mice received the electric stimuli, animals were monitored via video cameras and the locomotive response to these stimuli was measured for each animal.

Movements of the animals were detected by infrared sensors (distance: 14 mm). Freezing behavior was defined as no infrared beam crosses for more than 1 second. Additionally, distance traveled was automatically recorded during all phases of the experiments. A high correlation of the automatic measurement of freezing behavior of the TSE FC system and observer scoring of freezing has been repeatedly published.[24,25]

2.2.6 | Safety learning procedure

Ten days following stress exposure, the mice were submitted to the safety learning procedure which took place during the first hours of the light phase. Safety learning was performed in the TSE or Panlab fear conditioning systems described above. On day one, mice were habituated to the conditioning boxes for 3.5 minutes, with one presentation of a tone stimulus (10 kHz, 85 dB, 30 seconds), the to-be-learned safety cue. The following 2 days, safety conditioning was performed. For each session, mice were individually placed into the box and exposed to five explicit unpairings (inter-stimulus interval: 120 seconds) of the tone stimulus (conditioned stimulus: CS) and a scrambled electric stimulus (foot shock: 0.4 mA, 2 seconds). The minimal interval between a tone stimulus and an electric stimulus was 30 seconds. On day four, mice were tested for learned safety in an expression session. For this, mice were placed into the conditioning boxes for 7.5 minutes, with the safety CS being presented five times for 30 seconds (inter-stimulus interval: 60 seconds). The mean freezing response to the conditioning context 30 seconds before each CS presentation was quantified and compared to the mean freezing response during the safety CS.

2.3 | Descriptive and statistical analysis

For the safety learning procedure, the mean percent in freezing to the context and the tone stimulus (later safety CS) were calculated for the acclimation and expression sessions. Furthermore, the percent effect of the safety CS related to contextual freezing for each individual was calculated.

Statistical analyses were separately performed for male and female mice, using Prism 7.0 (GraphPad Software Inc., La Jolla, CA) and Systat 11 (Systat Software, San Jose, CA). All data were checked for normal distribution with the D'Agostino-Pearson omnibus normality test. Statistical significance for the individual percent effect of the safety CS related to contextual freezing was analyzed with Student's two-tailed t-Test. Percent context freezing and safety CS freezing (fear inhibition) during the acclimation and expression session was analyzed by multi-factorial analyses of variance (ANOVA) with stress condition (stress, nonstress), and genotype (experiment 3: NPSR +/+, NPSR +/−, NPSR −/−) as between-subject factors, and trial type (context, safety CS) as within-subjects factor. Between-subjects and within-subject post-hoc comparisons were made using Sidak's multiple comparisons test. Main effects and interactions were deemed significant with $P \leq .05$ for all statistical tests. Results are represented as mean ± SEM.

3 | RESULTS

3.1 | Exposure to immobilization stress does not affect conditioned safety

Ten days following exposure to the immobilization stress, C57BL/6J mice were submitted to an acclimation session in which freezing behavior to a novel context and a tone stimulus was evaluated.

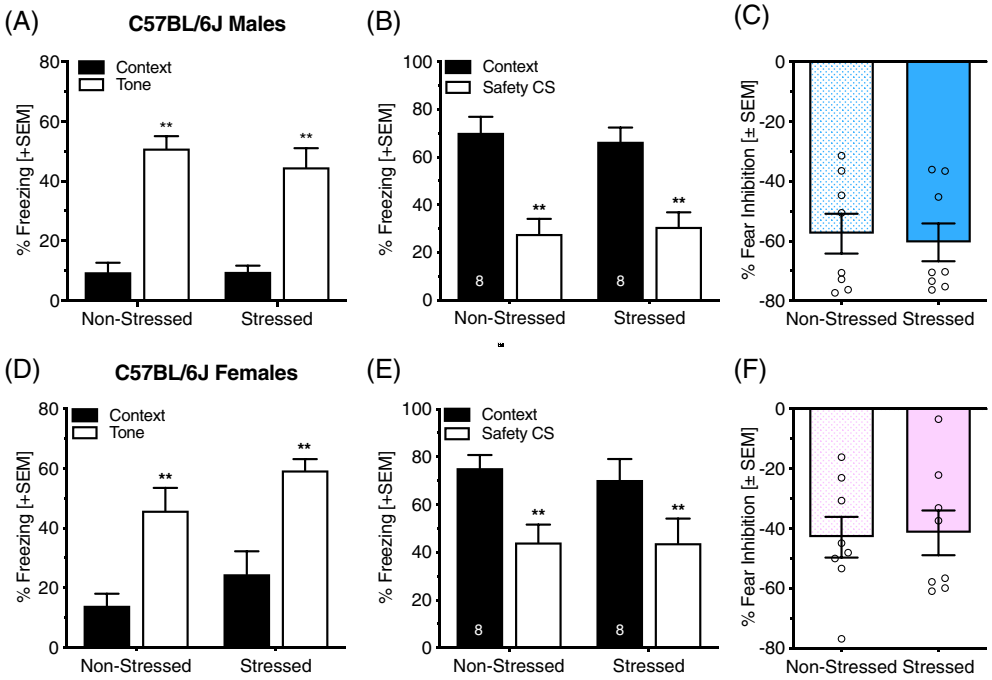

FIGURE 1 Pre-exposure to immobilization stress does not affect safety learning. During the acclimation session all mice, males (A) as well as females (D), significantly increased their freezing behavior to the tone in comparison to the novel context. During the expression session, stressed and nonstressed males (B, C), as well as stressed and nonstressed females (E, F), significantly reduced their freezing behavior during the safety CS. Data are represented as group averages ± SEM. Numbers depicted in the bars represent the n of each group. $^{**}P < .0001$

Nonstressed, as well as stressed male C57BL/6J mice significantly increased freezing upon tone presentation (Figure 1A; $F_{(1,\ 14)} = 140.80$, $P < .0001$). There was no main effect of stress condition ($F_{(1,\ 14)} = 0.004$, $P = .95$; interaction: $F_{(1,\ 14)} = 5.11$, $P = .04$). Post-hoc Sidak's multiple comparison test revealed that freezing behavior during the tone stimulus presentation was significantly higher in both groups (nonstressed: $t_{(14)} = 9.99$, $P < .0001$; stressed: $t_{(14)} = 6.79$, $P < .0001$), while there were no differences in contextual freezing ($t_{(28)} = 1.10$, $P = .48$). A similar behavioral pattern was observed in stressed and nonstressed female C57BL/6J mice (Figure 1D; ANOVA: trial type: $F_{(1,\ 14)} = 74.35$, $P < .0001$; stress condition: $F_{(1,\ 14)} = 2.87$, $P = .11$; interaction: $F_{(1,\ 14)} = 0.04$, $P = .84$; Sidak's post-hoc: nonstressed: $t_{(14)} = 6.24$, $P < .0001$; stressed: $t_{(14)} = 5.96$, $P < .0001$; context freezing: $t_{(28)} = 1.60$, $P = .23$).

Following the acclimation session, C57BL/6J mice were submitted to the safety conditioning protocol. In the expression session, male C57BL/6J mice significantly reduced freezing to the safety CS compared to freezing behavior to the context (Figure 1B; $F_{(1,\ 14)} = 131.40$, $P < .0001$; for time course, see Supplementary Information, Figure S1A). There was no main effect of immobilization stress ($F_{(1,\ 14)} = 0.0075$, $P = .99$; interaction: $F_{(1,\ 14)} = 0.03$, $P = .86$). Female C57BL/6J mice also reduced their freezing behavior to the safety CS (Figure 1E; $F_{(1,\ 14)} = 68.86$, $P < .0001$), regardless of stress condition ($F_{(1,\ 14)} = 0.21$, $P = .66$; interaction: $F_{(1,\ 14)} = 0.01$, $P = .91$).

These findings were confirmed by the analyses of the percent effect of the safety CS related to contextual freezing (Figure 1C; Figure 1F). This analysis revealed a main effect of sex (ANOVA: sex: $F_{(1,\ 28)} = 5.55$, $P = .03$) but not of the stress condition ($F_{(1,\ 28)} = 0.29$, $P = .59$; interaction: $F_{(1,\ 28)} = .01$, $P = .91$).

3.2 | Stress by exposure to mild electric stimuli increases freezing to a novel context and impairs conditioned safety in a sex-specific manner

Ten days following stress by exposure to electric stimuli that significantly increased plasma corticosterone levels (Supplementary Information, Figure S2), mice were submitted to an acclimation session in which freezing behavior to a novel context and a tone stimulus was evaluated. We found a significant effect of stress, with stressed male C57BL/6J mice showing significantly higher levels of freezing behavior to the novel context and the tone stimulus than their nonstressed conspecifics (Figure 2A; ANOVA: trial type: $F_{(1,\ 40)} = 0.05$, $P = .82$; stress condition: $F_{(1,\ 40)} = 76.14$, $P < .0001$; interaction: $F_{(1,\ 40)} = 0.09$, $P = .77$). Female C57BL/6J mice showed a similar behavioral pattern, with stressed females freezing significantly more in the novel context and to the tone stimulus than nonstressed mice (Figure 2D; ANOVA: trial type: $F_{(1,\ 39)} = 7.59$, $P = .009$; stress condition: $F_{(1,\ 39)} = 160.2$, $P < .0001$; interaction: $F_{(1,\ 39)} = 1.73$, $P = .20$). Post-hoc Sidak's multiple comparison test confirmed these findings (context: $t_{(78)} = 10.42$, $P < .0001$; tone: $t_{(78)} = 8.70$, $P < .0001$). Moreover, stressed female mice froze significantly less during the tone stimulus than to the context (Figure 2D; $t_{(39)} = 2.84$, $P = .01$), while there was no difference between freezing behavior to the context and the tone in nonstressed animals ($t_{(39)} = 1.03$, $P = .52$).

Following the acclimation session, C57BL/6J mice were submitted to the safety learning protocol. In the expression session, freezing during the safety CS presentations was significantly reduced compared to freezing behavior to the context in both, stress and nonstressed male C57BL/6J mice (Figure 2B; ANOVA: trial type: $F_{(1,\ 40)} = 188.70$, $P < .0001$; for time course, see Supplementary

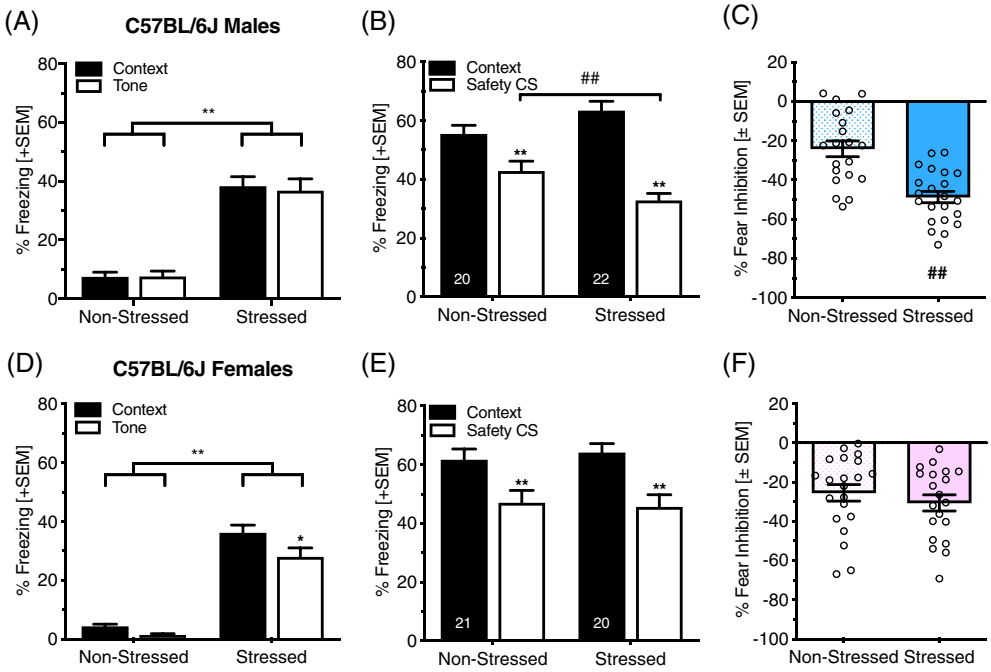

FIGURE 2 Pre-exposure to mild electric stimuli enhances safety learning in male mice. During the acclimation session both, stressed male (A) and female (D) mice, significantly increased their freezing behavior to the novel context and the tone in comparison to their nonstressed conspecifics. During the expression session, all mice significantly reduced their freezing behavior during the safety CS, demonstrating successful safety learning. In stressed male mice (B, C), the reduction of the freezing response by the safety CS was significantly stronger than the nonstressed males. (E, F) In female mice, pre-exposure to electric stimuli did not affect safety learning. Data are represented as group averages ± SEM. Numbers depicted in the bars represent the n of each group. *P < .01, ** P < .0001, post-hoc comparisons with contextual freezing or as indicated, ## P < .0001, comparison with nonstressed mice

Information, Figure S1B). There was no main effect of the stress condition ($F_{(1,\ 40)}$ = 0.06, P = .80) but a significant interaction between stress condition and trial type ($F_{(1,\ 40)}$ = 32.47 P < .0001). Post-hoc Sidak's multiple comparison test revealed that freezing behavior during the safety CS presentation was significantly lower in stressed than in nonstressed male C57BL/6J mice ($t_{[80]}$ = 2.26, P = .05), while contextual freezing did not differ ($t_{[80]}$ = 1.79, P = .15). This effect could not be observed in female C57BL/6J mice. Here, the freezing response to the safety CS was reduced in both groups in comparison to context freezing, but there was no interaction between stress condition and the trial type (Figure 2E; ANOVA: trial type: $F_{(1,\ 39)}$ = 113.3, P < .0001; stress condition: $F_{(1,\ 39)}$ = 0.01, P = .92; interaction: $F_{(1,\ 39)}$ = 1.56, P = .23; percent difference scores: Figure 2F; t-test: t (39) = 0.87, P = .39). Notably, further analysis revealed that stressed female mice decreased freezing to the safety CS significantly more in this expression session than during the acclimation (Supplementary Information, Figure S3; ANOVA: trial type: $F_{(1,\ 19)}$ = 27.11, P < .0001; session: $F_{(1,\ 19)}$ = 50.82, P < .0001; interaction: $F_{(1,\ 19)}$ = 6.86, P = .02), indicating successful safety learning.

Analysis of percent differences scores (Figure 2C & 2F) confirmed these findings and revealed main effects of sex and stress condition, as well as a significant interaction between sex and stress condition (ANOVA: stress condition: $F_{(1,79)}$ = 15.07, P = .0002; sex: $F_{(1,79)}$ = 4.76, P = .03; interaction: $F_{(1,79)}$ = 6.49, P = .01). Post-hoc Sidak's multiple comparison test showed a significant difference between stressed male and female C57BL/6J mice ($t_{[79]}$ = 3.36, P = .002), while there was no difference in nonstressed male and female C57BL/6J mice ($t_{[79]}$ = 0.26, P = .96).

3.3 | Neuropeptide S receptor deficiency interferes with sex-dependent modulation of safety learning by stress

Because we did not observe an effect of the immobilization stressor on safety learning, NPSR +/+, +/− and −/− mice were only submitted to pre-exposure to electric stimuli.

Evaluation of the acclimation session in stressed and nonstressed male (Figure 3A) and female mice (Figure 3D) revealed that, independent of NPSR genotype, stressed mice froze significantly more to the unfamiliar context and the tone stimulus than their nonstressed conspecifics (Males (Figure 3A); ANOVA: stress condition: $F_{(1,57)}$ = 82.40, P < .0001; interaction genotype x stress condition: $F_{(2,57)}$ = 2.15, P = .13; Females (Figure 3D); ANOVA: stress condition: $F_{(1,57)}$ = 178.36, P < .0001; interaction genotype x stress condition: $F_{(2,57)}$ = 2.37, P = .10). In both sexes, there were no genotype or CS effects (Males (Figure 3A): genotype: $F_{(2,57)}$ = 1.22, P = .30; trial type: $F_{(1,57)}$ = 0.05, P = .82; Females (Figure 3D): genotype: $F_{(2,57)}$ = 1.28, P = .29; trial type: $F_{(1,57)}$ = 0.01, P = .94).

Following the acclimation session, NPSR +/+, +/− and −/− mice were submitted to safety conditioning. During the expression session,

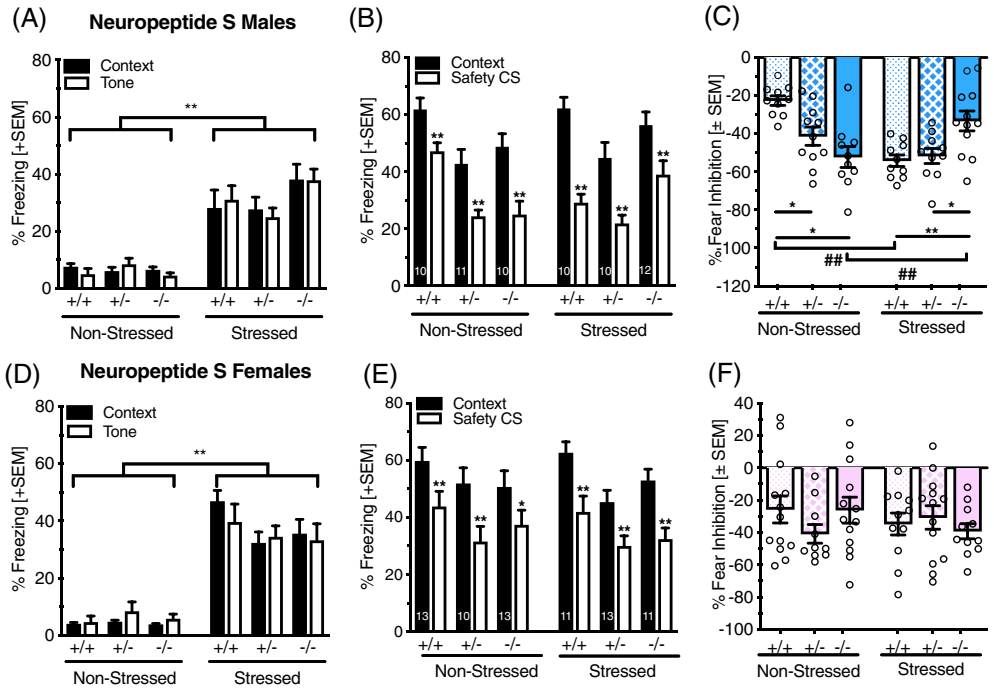

FIGURE 3 NPSR-deficiency prevents the beneficial effect of stress exposure on safety learning in male mice. (A, B) During the acclimation session, both, stressed male (A) and female (D) mice of all genotypes, significantly increased their freezing behavior to the novel context and the tone in comparison to their nonstressed conspecifics. (B,C) During the expression session of male mice, all mice significantly reduced their freezing behavior during the safety CS (B). Data further revealed that NPSR-deficiency enhanced safety learning in nonstressed male mice (C). Pre-exposure to electric stimuli increased safety learning in male NPSR +/+ mice, while this effect was blocked in NPSR-deficient male mice showing impaired, but still intact, safety learning (C). During the expression session of female mice, all mice significantly reduced their freezing behavior during the safety CS, without there being an effect of genotype or stress condition (E, F). Data are represented as group averages ± SEM. Numbers depicted in the bars represent the n of each group. *P < .01, ** P < .001, post-hoc comparisons with contextual freezing or as indicated, ## P < .001, comparison with nonstressed mice

male mice significantly reduced their freezing response to the safety CS, regardless of genotype or stress condition (Figure 3B; ANOVA: trial type: $F_{(1,57)}$ = 341.18, P < .0001; for time course, see Supplementary Information, Figure S4). Moreover, the ANOVA revealed a significant trial type × genotype × stress condition interaction (interaction: $F_{(2,57)}$ = 10.83, P < .0001; genotype: $F_{(2,57)}$ = 8.153, P = .001). The analysis of the percent effect of the safety CS confirmed this finding (Figure 3C; ANOVA: genotype: $F_{(2,57)}$ = 1.66, P = .20; stress condition: $F_{(1,57)}$ = 4.52, P = .04; interaction genotype x stress condition $F_{(2,57)}$ = 16.59, P < .0001). Post-hoc comparison tests revealed that nonstressed male NPSR −/− mice displayed significantly higher levels of fear inhibition by the safety CS than stressed NPSR −/− mice ($t_{[57]}$ = 3.10, P = .009). Analysis of the wildtype conspecifics revealed an opposite behavioral pattern (nonstressed NPSR +/+ vs −/− mice: $t_{(57)}$ = 4.65, P < .0001; stressed NPSR +/+ vs −/− mice: $t_{(57)}$ = 3.41, P = .004), with nonstressed NPSR +/+ males showing significantly lower levels of fear inhibition than their stressed conspecifics ($t_{(57)}$ = 4.95, P < .0001). Moreover, there was a significant difference between stressed male NPSR −/+ and −/− mice ($t_{(57)}$ = 3.01, P = .01), as well as between nonstressed NPSR +/+ and +/− mice ($t_{(57)}$ = 3.01, P = .01).

Female mice significantly reduced their freezing response during the safety CS, regardless of genotype or stress condition (Figure 3E; ANOVA: trial type: $F_{(1,65)}$ = 113.94, P < .0001; stress condition: $F_{(1,65)}$ = 0.51, P = .48; genotype: $F_{(2,65)}$ = 0.07, P = .93). There was no interaction of trial type, genotype and stress condition ($F_{(2,65)}$ = 1.26, P = .29). The analysis of the percent effect of the safety CS related to contextual freezing confirmed that neither stress condition, nor genotype influenced the expression of safety memory (Figure 3F; ANOVA: genotype: $F_{(2,65)}$ = 0.29, P = .75; stress condition: $F_{(1,65)}$ = 0.46, P = .50; interaction: $F_{(2,65)}$ = 1.45, P = .24).

An overall ANOVA including the data from male and female mice confirmed the sex difference of the stress effect on safety learning. This analysis revealed a main effect of sex ($F_{[1122]}$ = 7.33, P = .008), an interaction between genotype and stress condition ($F_{[2122]}$ = 4.18, P = .02) and most importantly an interaction between sex, genotype and stress condition ($F_{[2122]}$ = 6.39, P = .002). All other factors or interactions did not reach the level of statistical significance (Fs < 2.68, Ps > .10).

4 | DISCUSSION

The aim of the present study was to investigate whether NPSR-deficiency interferes with safety learning and its modulation by pre-exposure to a stressor. First, we explored whether and how safety

learning is modulated by pre-exposure to stress. For this, we exposed C57Bl/6J mice to two different kinds of stressors and investigated their effect on safety learning 10 days later. We found that there were no differences in safety learning following immobilization stress (Figure 1). In contrast, stress induced by electric stimuli enhanced safety learning in males, but had no effect on female C57Bl/6J mice (Figure 2). There were no sex differences in the absence of stress. Because the NPS system has been proposed to have a modulatory role in stress and cognitive functions, we next investigated whether mice with NPSR-deficiency show impairments in safety learning. We found that in male mice without pre-exposure to electric stimuli, NPSR-deficiency enhanced safety learning, while there was no effect in female mice (Figure 3). Pre-exposure to electric stimuli increased safety learning in male NPSR +/+ mice, thereby confirming the observations of our second experiment. However, this pre-exposure effect was blocked in NPSR-deficient male mice, showing significantly impaired, but still intact, safety learning in comparison to their NPSR +/+ and NPSR +/− littermates (Figure 3C). There was neither a pre-exposure nor a genotype effect in female mice (Figure 3F).

In our first experiment we investigated the effect of immobilization stress on safety learning in C57Bl/6J mice (Figure 1). Since immobilization stress has been described as a severe emotional stressor,[23] we expected to see long-term effects on safety learning. Surprisingly, we did not find any changes in safety learning after immobilization stress in our experiment. However, all mice of this experiment showed increased freezing to the tone in the acclimation session. This stands in contrast to our second experiment, in which the tone did not induce a freezing response in nonstressed mice while stressed mice showed increased freezing throughout the whole acclimation session. Notably, the test protocol, including the different parameters of the tone such as frequency, duration and intensity were identical in these two experiments. We assume that the animals' history could be responsible for this specific difference. Whereas the C57Bl/6J mice from experiment 2 were bred in the in-house animal facility (original breeding pairs from Charles River, Germany), C57Bl/6J mice from experiment 1 were directly obtained from Charles River Spain. Apart from the different living/raising conditions of the animals, this also implicates that the mice of experiment 1 experienced a transportation process, which may have caused additional stress and thereby altered findings.[26] Indeed, the life history of animals can influence fear responses later in life and adverse experiences, such as transport stress, can increase the responsiveness to a harmless stimulus,[27] such as the prospective tone CS in the acclimation session of our experiments. Sensitization by transport stress has previously been shown in BALB/c mice.[28] In this study, BALB/c mice from the local breeding facility showed a more resilient phenotype, whereas BALB/c mice obtained from a commercial breeder represented a more susceptible phenotype for developing exaggerated fear responses, including differences in amygdala long-term depression and surface GluR1 trafficking.[28] In our experiment, such transport-related sensitization may have masked potential effects of the immobilization stressor. However, whereas the tone seemed to be somewhat aversive to all mice in experiment 1, stressed mice did not display increased freezing to the novel context, as observed in the stressed mice of experiment 2. Independent of these behavioral differences during the acclimation session, the mice in experiment 1 were able to learn conditioned safety, that is, the contextual freezing during the retention test was efficiently suppressed by the safety CS. Thus, the pre-exposure to immobilization stress had no effect on safety learning in the expression session. Notably, a variety of studies have shown that immobilization stress induces multiple PTSD-like symptoms that last longer than 24-48 hours, such as impaired declarative memory, impaired fear extinction or enhanced anxiety.[23,29-31] Since patients suffering from PTSD have been shown to exhibit impaired safety learning,[14,15] we also expected impaired safety learning in the mice pre-exposed to immobilization stress. One possible explanation for the missing effect of immobilization stress could be that the mice in this experiment generally showed very efficient safety learning, so that the protocol used was not sensitive to disturbing influences any more. Therefore, we cannot exclude that a more sensitive safety learning protocol would be affected by immobilization stress. Another explanation could be that the effects of immobilization stress are limited to a specific time window (< 7 days) and our safety learning experiment was performed outside this critical time window.

In our second experiment, we investigated the effect of stress by exposure to electric stimuli on safety learning in C57Bl/6J mice (Figure 2; Supplementary Information, Figure S2). Ten days following stress exposure, mice were submitted to the safety learning protocol. In the acclimation session, stressed male and female C57Bl/6J mice showed significantly increased freezing behavior to the context, as well as to the tone compared to their nonstressed conspecifics (Figure 2A,D). This increased freezing response may be due to sensitization and/or fear generalization, induced by the pre-exposure to the electric stimuli. In order to survive, individuals use their past experiences to predict future encounters that are similar to a previously aversive incident. Because these events are never identical, a flexible assessment of a potentially threatening stimulus or context via generalization of fear is of high advantage.[32] We further observed that female mice slightly decreased their freezing response during presentation of the tone (Figure 2D). This sex difference is in line with the concept that fears generalization can be modulated by numerous external and intrinsic factors including sex.[32] During the expression session, all animals, regardless of sex or stress condition, learned safety (Figure 2B,C; Figure 2E,F; Supplementary Information, Figure S3), as represented by decreased freezing behavior in the presence of the safety CS. Importantly, stressed male C57Bl/6J mice learned safety significantly better than their nonstressed conspecifics or females. One reason for this finding could be that the previous experience with the electric stimuli during the pre-exposure session de-sensitized the mice so that they were less stressed by the electric stimuli during safety conditioning and, therefore, more susceptible to acquire safety learning. In fact, a similar phenomenon has previously been described by Solomon and Corbit (1978)[33] in their "Opponent-Process Theory of Motivation". According to this theory, every motivationally relevant stimulus induces two opponent processes. For example, an aversive stimulus first induces fear, which is then -upon

termination- followed by an opponent emotion, such as relief or safety. Solomon and Corbit predicted that with repeated exposures to this stimulus, the system adapts and the opponent emotion may be facilitated. Translating this to our experiment, it seems that at least our male mice adapted to the electric stimuli by the pre-exposure and, therefore, safety response after the offset of the electric stimuli in the safety conditioning sessions was more pronounced. This, in turn, may facilitate safety learning, an observation that could not be found in female mice. Reason for the observed sex difference could be that females often show higher trait anxiety than males[34-37] and that trait anxiety is negatively correlated with the "opponent" emotion safety.[38]

Taken together, our first two experiments demonstrated that safety learning seems to be a very robust type of learning that cannot be impaired by pre-exposure to stress, at least with the protocol used here. Pre-exposure to immobilization stress did not affect behavior in the acclimation phase as well as safety learning per se. This was different after pre-exposure to electric stimuli. Here, an increased freezing response during the acclimation phase, that is, to a novel context, was induced, suggesting that the electric stimuli induce a sensitization and/or fear conditioning to parts of this context. For example, the mice were already familiar with the floor grid that transmitted the electric stimuli and, hence, stressed animals could direct their focus more towards the learning of the safety cue. However, safety learning was facilitated by pre-exposure to electric stimuli in male mice only. The improvement in safety learning by pre-exposure to electric stimuli could be based on opponent motivational processes (see discussion above) since animals were pre-exposed to the same aversive stimuli as the one used in the safety conditioning protocol (homotypic stressors), whereas immobilization stress represents heterotypic stressors.

In our third experiment we investigated the effect of NPSR-deficiency on safety learning and its modulation by pre-exposure to electric stimuli (Figure 3). Because we did not observe an effect of immobilization stress on safety, we waive testing NPSR-deficient mice in this paradigm. During the acclimation session, all stressed mice showed increased freezing behavior in the novel context, which confirms our findings from experiment 2. During the expression session, all animals significantly reduced freezing to the safety CS, indicating that they acquired learned safety. In line with the findings of our second experiment, stressed male NPSR +/+ mice learned safety significantly better than their nonstressed conspecifics. This facilitation of safety learning was not seen in stressed male NPSR −/− mice. In contrast, nonstressed NPSR −/− males learned safety significantly better than nonstressed male NPSR +/+ mice. Both stressed and nonstressed male NPSR +/− mice expressed an intermediate phenotype. Notably, the NPSR genotype neither affects reactivity to the electric stimuli during safety conditioning (see Supplementary Information, Figure S5B and 5C) nor the corticosterone response to electric stimuli as previously shown.[39,40]

Human, as well as rodent studies investigating the role of the NPS system in anxiety-related behaviors have come to the general consensus that NPS plays an important role in the regulation of anxiety, fear and stress-related behaviors.[17-21] Generally, NPSR-deficient animals show a more anxious phenotype but not impaired contextual or cued fear learning.[39,41,42] Here we show that NPSR-deficiency leads to improved safety learning in mice which were not pre-exposed to electric stimuli, that is, NPSR-deficiency has beneficial effects in mice that have not encounter an aversive experience. However, in pre-exposed mice, NPSR-deficiency prevented the facilitating effects of this pre-exposure on safety learning. How can these two very different effects of NPSR-deficiency be explained? As pointed out above, NPSR −/− mice express more trait anxiety[41,42] and, interestingly, the increase of anxiety after fear conditioning in NPSR-deficient mice is driven by the female mice.[39] However, at least in male rats, increased trait anxiety is negatively correlated with safety learning.[38] The present data suggests that stress pre-exposure or higher trait anxiety (as shown in NPSR-deficient mice) increases the stress reactivity of an individual, which may follow an inverted U-shape association with safety learning. While having either higher trait anxiety or pre-exposure to a mild stressor separately may exert an "optimal" level of reactivity that promotes safety learning, a combination of both, higher trait anxiety paired with pre-exposure to stress, would not have beneficial effects on safety learning. This would explain why we observed that either pre-exposure to electric stimuli in C57Bl/6J and NPSR +/+ mice, or NPSR-deficiency, leads to beneficial effects on safety learning. However, if NPSR-deficient animals were pre-exposed to electric stimuli, this adds up to an over-optimal emotional state at which safety learning is not affected anymore. The same over-optimal state may be induced by a pre-exposure to a more severe stressor (experiment 1) or by more increased trait anxiety (female mice in all experiments).

As discussed above, a variety of studies have described a human NPSR polymorphism that is associated with an increased prevalence of anxiety disorders. If we would translate the above-described inverted U-shape relationship to human studies, this would implicate that subjects with the NPSR risk polymorphism may have an advantage until they encounter too many aversive or stressful situations. It would be of high interest to investigate whether safety learning in humans is also dependent on the NPSR genotype, sex or pre-exposure to stressful life events.

In conclusion, our findings demonstrate a facilitating effect of pre-exposure to electric stimuli on safety learning in male mice. This beneficial pre-exposure effect on safety learning was not observed in females, independent of genotype, or male NPSR −/− mice. One explanation for this interesting phenomenon may be an inverted U-shape relationship between pre-exposure and/or trait anxiety (i.e., determined by genotype and/or sex) and safety learning. Future research in humans and rodents should consider such complex relationships and therefore additionally evaluate factors potentially influencing these relationships.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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