Cannabinoids Prevent the Development of Behavioral and Endocrine Alterations in a Rat Model of Intense Stress

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Cannabinoids have recently emerged as a possible treatment of stress- and anxiety-related disorders such as post-traumatic stress disorder (PTSD). Here, we examined whether cannabinoid receptor activation could prevent the effects of traumatic stress on the development of behavioral and neuroendocrine measures in a rat model of PTSD, the single-prolonged stress (SPS) model. Rats were injected with the CB1/CB2 receptor agonist WIN55,212-2 (WIN) systemically or into the basolateral amygdala (BLA) at different time points following SPS exposure and were tested 1 week later for inhibitory avoidance (IA) conditioning and extinction, acoustic startle response (ASR), hypothalamic-pituitary-adrenal (HPA) axis function, and anxiety levels. Exposure to SPS enhanced conditioned avoidance and impaired extinction while enhancing ASR, negative feedback on the HPA axis, and anxiety. WIN (0.5 mg/kg) administered intraperitoneally 2 or 24 h (but not 48 h) after SPS prevented the trauma-induced alterations in IA conditioning and extinction, ASR potentiation, and HPA axis inhibition. WIN microinjected into the BLA (5 µg/side) prevented SPS-induced alterations in IA and ASR. These effects were blocked by intra-BLA co-administration of the CB1 receptor antagonist AM251 (0.3 ng/side), suggesting the involvement of CB1 receptors. These findings suggest that (i) there may be an optimal time window for intervention treatment with cannabinoids after exposure to a highly stressful event, (ii) some of the preventive effects induced by WIN are mediated by an activation of CB1 receptors in the BLA, and (iii) cannabinoids could serve as a pharmacological treatment of stress- and trauma-related disorders.
Neuropsychopharmacology (2012) **37,** 456–466; doi:10.1038/npp.2011.204; published online 14 September 2011

Keywords: CB1 receptors; hypothalamic-pituitary-adrenal (HPA) axis; inhibitory avoidance extinction; single prolonged stress; basolateral amygdala; post-traumatic stress-disorder

INTRODUCTION

The cannabinoid system is part of the complex circuitry that regulates anxiety and stress and is a crucial mediator of emotional learning (Marsicano *et al*, 2002; Viveros *et al*, 2005; Patel *et al*, 2005, 2005a; Laviolette and Grace, 2006; Varvel *et al*, 2007; Ganon-Elazar and Akirav, 2009; Lutz, 2009; Hill *et al*, 2009; Abush and Akirav, 2010; Akirav, 2011). Recently, it has been suggested that the cannabinoid system could represent a therapeutic target for the treatment of stress- and anxiety-related disorders such as post-traumatic stress disorder (PTSD) (Porter and Felder, 2001; Marsicano *et al*, 2002; Kathuria *et al*, 2003). In humans, potential benefits of the synthetic cannabinoid nabilone were demonstrated in PTSD patients (Fraser, 2009).

The single-prolonged stress (SPS) model (Liberzon *et al*, 1997) is a valuable tool to examine the neural and endocrine circuitry related to the effects of intense stress on fear

learning and regulation of stress responsivity that are relevant to PTSD. Exposure to SPS involves three different stress paradigms and, after an undisturbed period of 7 or 14 days, rats show enhanced negative feedback on the hypothalamic-pituitary-adrenal (HPA) axis and an exaggerated acoustic startle response (ASR) (Liberzon *et al*, 1997; Khan and Liberzon, 2004; Kohda *et al*, 2007). Enhanced ASR and HPA-negative feedback have been reliably observed in patients with PTSD (Shalev *et al*, 1992; Yehuda *et al*, 1993; Orr *et al*, 1997).

SPS exposure also results in impaired extinction of contextual fear (Yamamoto *et al*, 2007). Impaired fear extinction is a major symptom of anxiety disorders caused by emotional trauma, such as PTSD. Moreover, it has been suggested that the continued ability of conditioned stimuli to elicit traumatic memories and flashbacks in PTSD results from a deficit in the neural mechanisms involved in extinction (Charney *et al*, 1993). PTSD patients also demonstrate impaired extinction in the aftermath of the trauma (Orr *et al*, 2000; Milad *et al,* 2008). For example, Milad *et al* (2008) have shown deficient extinction recall as measured in skin conductance response in a 2-day fear conditioning and extinction procedure in PTSD patients.

We have recently found that cannabinoid receptor activation in the basolateral amygdala (BLA) using the

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CB1/2 receptor agonist WIN55,212-2 (WIN) can prevent the stress-induced enhancement of inhibitory avoidance (IA) conditioning as well as the stress-induced disruption of IA extinction. This reversal effect was found to be associated with alterations in the HPA axis, as intra-BLA WIN inhibited the stress-induced increase in plasma corticosterone (CORT) levels (Ganon-Elazar and Akirav, 2009). Other studies have suggested that, in non-stressed rats, cannabinoid systemic activation facilitates fear extinction (Chhatwal *et al*, 2005). We have recently shown that WIN administered into the CA1 facilitates IA extinction, with no effect on conditioned avoidance (Abush and Akirav, 2010).

In the current study, we aimed to examine whether cannabinoid receptor activation, using WIN administered systemically or into the BLA, could prevent SPS-induced alterations in IA conditioning and extinction, ASR, HPA axis function, and anxiety levels.

MATERIALS AND METHODS

Subjects

A total of 637 male Sprague Dawley rats (~ 60 days old, 250–300 g) were used for the experiments. Animals were caged individually at $22 \pm 2\,^{\circ}\text{C}$ under 12 h light/dark cycles (lights turned on at 0700 h and turned off at 1900 h). Rats had access to water and laboratory rodent chow *ad libitum*. All experiments were carried out between 0900 and 1500 h.

The experiments were approved by the University of Haifa Ethics and Animal Care Committee, and adequate measures were taken to minimize pain or discomfort.

Drug Treatment

WIN (i.p.: 0.5 mg/kg or 3 mg/kg; intra-BLA: 5 µg/side) and the CB1 receptor antagonist AM251 (0.3 ng/side) (Tocris Bioscience) were dissolved in dimethylsulfoxide (DMSO) and then diluted with saline (0.9% NaCl) and Tween-80 to achieve the final volume. Controls were given the vehicle only (1% DMSO, 1% Tween-80, and 98% saline). Drug concentrations were based on reports in the literature and our previous results (Martin *et al*, 1999; Ganon-Elazar and Akirav, 2009; Campolongo *et al*, 2009; Abush and Akirav, 2010; Segev and Akirav, 2011).

Cannulation and Drug Microinjection

Rats were anesthetized with 4.8 ml/kg Equithesin (2.12% w/v MgSO$_4$, 10% ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate) and implanted bilaterally with a stainless steel guide cannula (23 gauge, thin walled) aimed at the BLA (anteroposterior, -3 mm; lateral, ± 5 mm; ventral, -6.7 mm). Animals were allowed 1 week to recuperate before being subjected to experimental manipulations. Microinjection was performed bilaterally in a 0.5-µl volume per side delivered over 1 min. The injection cannula was left in position for an additional 60 s before withdrawal to minimize dragging of the injected liquid along the injection tract. The injection cannula was connected via polyethylene PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (PHD1000, Harvard Apparatus).

Single-Prolonged Stress

Rats were (i) restrained (7 cm diameter, 21 cm length) for 2 h, (ii) individually placed in a clear acrylic cylinder (20 cm diameter) filled to two thirds (35 cm) of its height with water (24 °C) and forced to swim for 15 min, and (iii) following 15 min recuperation, exposed to the inhalation anesthetic isoflurane (Nicholas Piramal) until the loss of consciousness. A cotton ball soaked in isoflurane was placed in a transparent test tube (to avoid any skin irritation to the rat caused by contact with the soaked cotton) that was placed near the rat's nose until deep anesthesia (indicated by a 50% reduction in respiratory rate and loss of the righting reflex). Control rats remained in a room adjacent to the SPS rats for the duration of SPS and were handled twice for several minutes.

Light-Dark IA

Described in Ganon-Elazar and Akirav (2009). Briefly, animals were placed in the light side of the IA apparatus. For conditioning (*Cond*), when the rat crossed over to the dark side of the box it received a 2 s, 0.7 mA scrambled footshock. After administration of the footshock, the opening between the two sides of the box was blocked, and the rats remained in the dark side for an additional 60 s, after which they were removed back to the home cage.

For extinction, rats were submitted to a non-reinforced test trial every 24 h for 3 days (*Ext1–Ext3*), beginning 24 h after conditioning. The latency to cross over to the dark side was measured. If, after 180 s, the rat did not cross over on its own, the experimenter gently guided it to the dark side. The opening between the two sides of the shuttle was then blocked, no footshock was administered, and the rat was allowed to explore the dark side freely for 180 s, after which it was removed back to the home cage.

Acoustic Startle Response

An acrylic animal holder (9 cm in diameter and 20 cm in length) connected to a piezoelectric accelerometer was placed in a sound proof chamber ($25 \times 25 \times 25$ cm). A high-frequency loudspeaker inside the chamber produced both continuous background noise (68 dB) and acoustic stimuli. Illumination was provided by a white bulb located on the ceiling of the chamber. The animals were placed in the holder and a startle session started following a 5-min habituation period. Sound stimuli consisting of a 50-ms burst of 120 dB white noise were delivered 30 times every 30 s. The background noise level of 68 dB was maintained throughout each session. The maximal amplitudes of ASR (%) were measured during a 1-s interval from the second the sound stimuli were delivered and were transferred to a computer using Harvard software (Panlab, Barcelona, Spain). The system allows recording and analysis of the signal generated by the animal movement through a high sensitivity Weight Transducer System. The maximal startle reflex response for each animal was

calculated as the average of the responses to the 30 auditory stimuli.

Open Field

The floor was white and divided by 1 cm wide black lines into 25 squares measuring 10×10 cm each ($50 \times 50 \times 38$) and placed under dim red light (<10 lux) (Ganon-Elazar and Akirav, 2009). Recordings were made of the time the rat spent in the central and the peripheral squares and the total distance covered over a period of 5 min. The open-field arena was thoroughly cleaned between each trial.

Light-Dark Test

Rats were placed in a box divided into two sides that were connected to each other through a small opening. One side was dark (black walls) and the other side was brightly lit (white walls; 80–90 lux). The rat was placed in the dark compartment facing away from the opening to the light side, and the number of entries into the light side and time spent in the light side were measured for 5 min.

Corticosterone Measurements and Dexamethasone Suppression Test

To test alterations in resting CORT levels following SPS, CORT was measured 2 min, 2, 24 h, or 1 week after SPS exposure.

To assess HPA-negative feedback, we used the dexamethasone (Dex) suppression test (DST) (Kohda *et al*, 2007). Rats were exposed to SPS and then administered with vehicle or with WIN at different time points. One week after SPS, Dex (0.05 mg/kg; Sigma, IL) was administered subcutaneously 2 h before a second stress exposure (elevated platform (EP), see below).

Trunk blood was collected after decapitation between 0900 and 1100 h. Samples were centrifuged at 3000 r.p.m. for 20 min at 4 °C. Serum was stored at −80 °C and analyzed for CORT using ELISA kits (DSL).

Elevated Platform

An EP (12×12 cm) stressor was used as the second stress exposure in the DST. Individual animals were placed on an EP for 30 min (height: 150 cm). The EP elicits stress responses in the form of behavioral 'freezing,' that is, immobility for up to 10 min, defecation, and urination (Maroun and Akirav, 2008; Ganon-Elazar and Akirav, 2009).

Histology

Following completion of the behavioral experiments, animals were deeply anesthetized and microinjected into the BLA with 0.5 μl of India ink. Brains were removed and brain slices (60 μm) were examined under a light microscope following Nissl staining to verify the cannula location. Placements of the cannulae were found to be incorrect in <10% of injected rats. Only data from animals with correct cannula placements were included in the analyses (Figure 1).
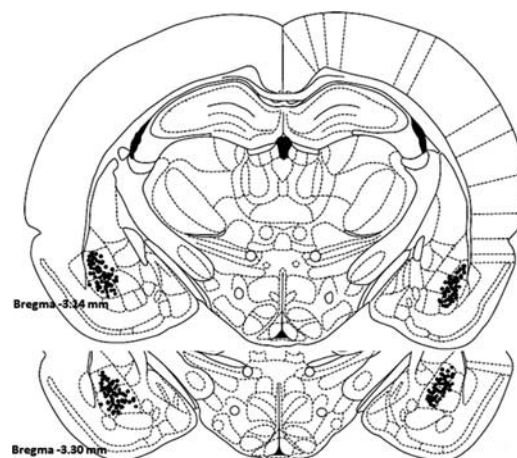

Figure I Representative schematic drawings of cannulae tip positions in the BLA. Solid black circles indicate cannula locations in a subset of animals (not all animals are shown in light of the large number of rats involved in the experiments). Two coronal views from positions 3.14 and 3.30 mm posterior to bregma.

Statistical Analysis

The results are expressed as mean values ± SEM. For statistical analysis, *t*-test, one-way ANOVA, and mixed design ANOVA were used. All *post hoc* comparisons were made using the least significant difference multiple-comparison test (LSD). Values are reported as mean values ± SEM.

RESULTS

The Effects of SPS and WIN55,212-2 Administered Systemically on IA

In our first experiment, we examined whether the cannabinoid receptor agonist WIN administered systemically (0.5 mg/kg) 2 min, 2, 24, or 48 h after SPS exposure would prevent the effects of SPS on conditioned avoidance and extinction tested a week later (Figure 2; data are shown in four different panels due to the number of groups involved).

When WIN was administered 2 min after SPS (Figure 2a), mixed ANOVA (groups × days (3×4)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,28)} = 6.10$; $p = 0.006$). A significant within-subject difference in the latency between the days was found ($F_{(1,28)} = 22.31$; $p = 0.0001$) but no significant interaction ($F_{(2,28)} < 1$; NS). One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,28)} = 7.28$; $p = 0.003$), Ext1 ($F_{(2,28)} = 4.48$; $p = 0.02$), and Ext2 ($F_{(2,28)} = 3.59$; $p = 0.041$). The SPS + Veh group demonstrated increased latency compared with the two other groups on Cond (Veh: $p = 0.002$; SPS + WIN2 min: $p = 0.005$) and Ext2 (Veh: $p = 0.026$; SPS + WIN2 min: $p = 0.043$). The Veh group demonstrated decreased latency compared with the two other groups on Ext1 (SPS + Veh: $p = 0.01$; SPS + WIN2 min: $p = 0.01$). This suggests that SPS rats (SPS + Veh and SPS + WIN2 min)
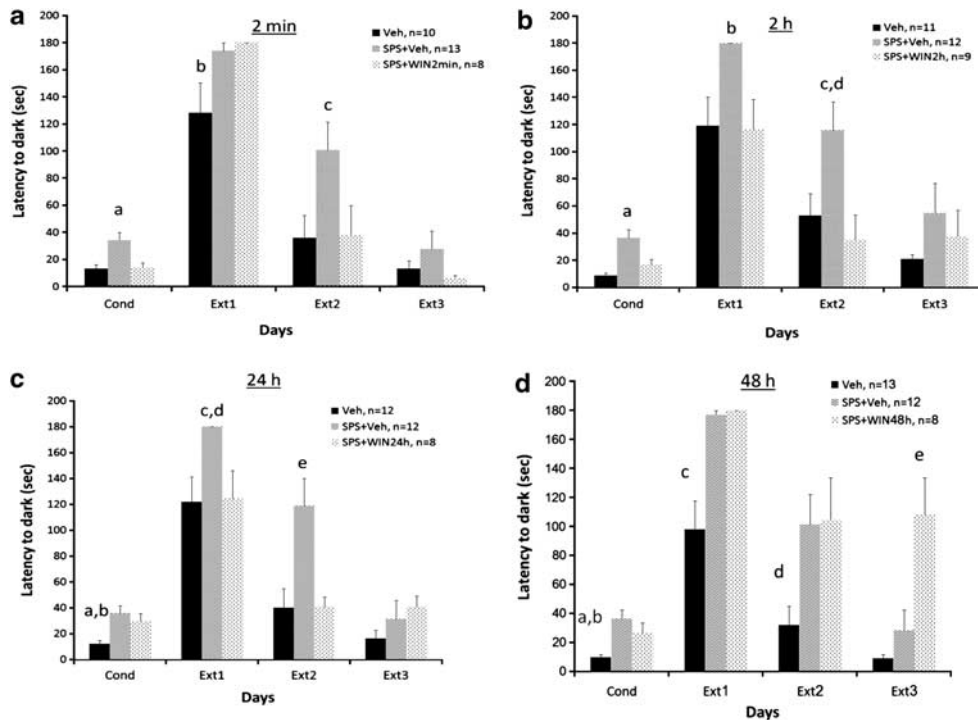
Figure 2 The effects of single-prolonged stress (SPS) exposure and systemic WIN55,212-2 administration on inhibitory avoidance (IA) conditioning and extinction. Rats were administered with vehicle (SPS + Veh) or WIN (SPS + WIN; 0.5 mg/kg) 2 min, 2, 24, or 48 h after SPS exposure and compared with a non-stressed group (Veh). The SPS + Veh groups demonstrated a significantly longer latency until they crossed over to the dark side of the IA apparatus on conditioning (Cond) day, fear retrieval (Ext1) and extinction (Ext2) than the Veh groups. (a) WIN administered 2 min after SPS exposure prevented the SPS-induced alterations in extinction (a, $p < 0.01$; c, $p < 0.05$: compared with Veh and SPS + WIN2 min; b, $p = 0.01$: compared with SPS + Veh and SPS + WIN2 min). (b) WIN administered 2 h after SPS exposure prevented the SPS-induced alterations in conditioned avoidance and extinction (a, $p < 0.01$; b, $p < 0.05$: compared with Veh and SPS + WIN2 h; c, $p < 0.05$: compared with Veh; d, $p < 0.01$: compared with SPS + WIN2 h). (c) WIN administered 24 h after SPS exposure prevented the SPS-induced alterations in conditioned avoidance and extinction (a, $p < 0.01$: compared with SPS + Veh; b, $p < 0.05$: compared with SPS + WIN24 h; c, $p < 0.01$: compared with Veh; d, $p < 0.05$: compared with SPS + WIN24 h; e, $p < 0.01$: compared with Veh and SPS + WIN24 h). (d) WIN administered 48 h after SPS exposure did not prevent the SPS-induced alterations in fear retrieval or extinction (a, $p < 0.01$: compared with SPS + Veh; b, $p < 0.05$: compared with SPS + WIN48 h; c, $p < 0.01$; d, $p < 0.05$: compared with SPS + Veh and SPS + WIN48 h; e, $p < 0.01$: compared with Veh and SPS + Veh).

show enhanced fear retrieval than the Veh group (Ext1) and that WIN administered 2 min post-SPS prevented the impairing effects of stress on extinction (Ext2).

When WIN was administered 2 h after SPS (Figure 2b), mixed ANOVA (groups × days (3 × 4)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,29)} = 5.93$; $p = 0.007$). There was no within-subject difference in the latency between the days ($F_{(1,29)} < 1$; NS), nor was there an interaction effect ($F_{(2,29)} < 1$; NS). However, analyzing the days of extinction (Ext2–3) revealed a significant interaction effect ($F_{(2,29)} = 3.34$; $p = 0.049$), further suggesting that exposure to SPS impaired extinction. One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,29)} = 11.21$; $p = 0.001$), Ext1 ($F_{(2,29)} = 4.77$; $p = 0.016$), and Ext2 ($F_{(2,29)} = 4.59$; $p = 0.019$). The SPS + Veh group demonstrated increased latency compared with the two other groups on Cond (Veh: $p = 0.001$; SPS + WIN2 h: $p = 0.004$), Ext1 (Veh: $p = 0.013$; SPS + WIN2 h: $p = 0.014$), and Ext2 (Veh: $p = 0.029$; SPS + WIN2 h: $p = 0.009$). This suggests that WIN administered 2 h post-SPS prevented the effects of stress on fear retrieval (Ext1) and extinction (Ext2).

When WIN was administered 24 h after SPS (Figure 2c), mixed ANOVA (groups × days (3 × 4)) revealed a significant

difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,29)} = 9.13$; $p = 0.001$). A significant within-subject difference in the latency between the days was found ($F_{(1,29)} = 6.31$; $p = 0.018$) but no significant interaction ($F_{(2,29)} < 1$; NS). However, analyzing the days of extinction (Ext2–3) revealed a significant interaction effect ($F_{(2,29)} = 7.08$; $p = 0.003$), further suggesting that exposure to SPS impaired extinction. One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,29)} = 6.91$; $p = 0.003$), Ext1 ($F_{(2,29)} = 4.69$; $p = 0.017$), and Ext2 ($F_{(2,29)} = 7.24$; $p = 0.003$). The Veh group demonstrated decreased latency compared with the two other groups on Cond (SPS + Veh: $p = 0.001$; SPS + WIN24 h: $p = 0.023$). The SPS + Veh group demonstrated increased latency compared with the two other groups on Ext1 (Veh: $p = 0.009$; SPS + WIN24 h: $p = 0.024$) and Ext2 (Veh: $p = 0.002$; SPS + WIN24 h: $p = 0.005$). This suggests that WIN administered 24 h post-SPS prevented the effects of stress on fear retrieval (Ext1) and extinction (Ext2).

When WIN was administered 48 h after SPS (Figure 2d), mixed ANOVA (groups × days (3 × 4)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(2,30)} = 15.99$; $p = 0.0001$).

There was no within-subject difference in the latency between the days but there was a significant interaction ($F_{(2,30)} = 6.91$; $p = 0.003$). Also, analyzing the days of extinction (Ext2–3) revealed a significant interaction effect ($F_{(2,30)} = 3.79$; $p = 0.034$). One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,30)} = 9.06$; $p = 0.001$), Ext1 ($F_{(2,30)} = 12.41$; $p = 0.001$), Ext2 ($F_{(2,30)} = 4.47$; $p = 0.02$), and Ext3 ($F_{(2,30)} = 7.50$; $p = 0.002$). The Veh group demonstrated decreased latency compared with the two other groups on Cond (SPS + Veh: $p = 0.001$; SPS + WIN48 h: $p = 0.026$), Ext1 (SPS + Veh: $p = 0.001$; SPS + WIN48 h: $p = 0.001$), and Ext2 (SPS + Veh: $p = 0.014$; SPS + WIN48 h: $p = 0.021$). The SPS + WIN48 h group demonstrated increased latency compared with the two other groups on Ext3 (Veh: $p = 0.001$; SPS + Veh: $p = 0.001$). This suggests that WIN administered 48 h post-SPS did not prevent the effects of stress on fear retrieval or extinction.

The Effects of SPS and WIN55,212-2 Administered into the BLA on IA

We have recently shown (Ganon-Elazar and Akirav, 2009) that WIN could prevent the effects of acute stress on IA conditioning and extinction when microinjected into the BLA.

In the current study, we examined whether intra-BLA WIN would prevent the effects of SPS exposure on IA conditioning and extinction and whether this effect is mediated by the CB1 receptor (Figure 3). Rats were exposed to SPS and treated with intra-BLA vehicle or WIN 2 min (Figure 3a) or 2 h (Figure 3b) after SPS exposure. Another set of rats was treated with Vehicle, WIN, AM251, or a combination of WIN + AM251 2 min after SPS exposure (Figure 3c). All rats were tested in the IA paradigm 1 week after SPS.

When WIN was administered into the BLA 2 min after SPS (Figure 3a), mixed ANOVA (groups × days ($3 × 4$)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(1,29)} = 125.03$; $p = 0.0001$). There was a within-subject difference in the latency between the days ($F_{(1,29)} = 7.30$; $p = 0.011$), with no significant interaction ($F_{(2,29)} < 1$; NS). One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,29)} = 4.33$; $p = 0.023$), Ext1 ($F_{(2,29)} = 4.25$; $p = 0.024$), and Ext3 ($F_{(2,29)} = 3.21$; $p = 0.05$). The SPS + Veh BLA group demonstrated increased latency compared with the Veh BLA group on Cond ($p = 0.007$). The SPS + Veh BLA group demonstrated increased latency compared with the two other groups on Ext1 (Veh BLA: $p = 0.035$; SPS + WIN2 min BLA: $p = 0.012$) and Ext3 (Veh BLA: $p = 0.04$; SPS + WIN2 min BLA: $p = 0.039$). This suggests that WIN administered into the BLA 2 min post-SPS prevented the effects of stress on fear retrieval (Ext1) and extinction (Ext3).

When WIN was administered into the BLA 2 h after SPS (Figure 3b), mixed ANOVA (groups × days ($3 × 4$)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(1,29)} = 231.65$; $p = 0.0001$). There was a within-subject difference in the
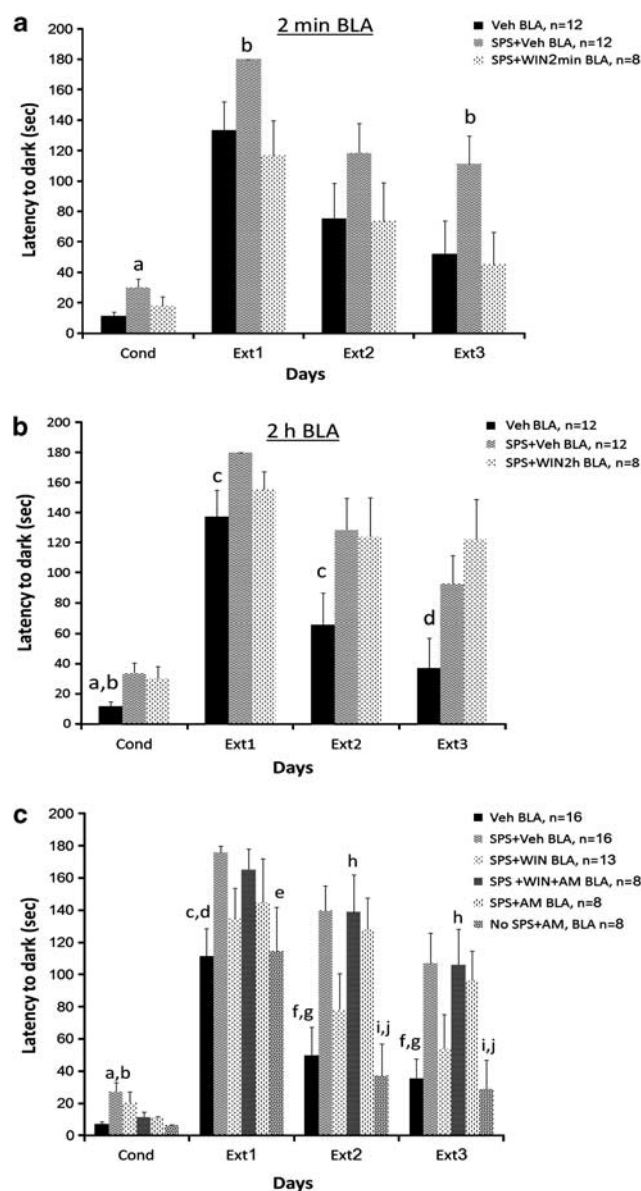

Figure 3 The effects of single-prolonged stress (SPS) exposure and intra-BLA WIN55,212-2 administration on inhibitory avoidance conditioning and extinction. Rats were administered with vehicle or WIN (5 μg/side) or AM251 (0.3 ng/side) or a combination of WIN + AM251 into the BLA. (a) WIN administered into the BLA 2 min after SPS exposure prevented the SPS-induced alterations in conditioned avoidance and extinction (a, $p < 0.01$: compared with Veh BLA; b, $p < 0.05$: compared with Veh BLA and SPS + WIN2 min BLA). (b) WIN administered into the BLA 2 h after SPS exposure did not prevent the SPS-induced alterations in conditioned avoidance and extinction (a, $p < 0.01$: compared with SPS + Veh BLA; b, $p < 0.05$: compared with SPS + WIN2 h BLA; c, $p < 0.05$: compared with SPS + Veh BLA; d, $p < 0.05$: compared with both groups). (c) AM251 (0.3 ng/side), co-administered with WIN (5 μg/side) into the BLA after SPS prevented the effects of WIN on SPS-induced alterations in extinction (a, $p < 0.01$: different from Veh and NO SPS + AM; b, $p < 0.05$: different from SPS + WIN + AM and SPS + AM; c, $p < 0.01$ different from SPS + Veh; d, $p < 0.05$: different from SPS + WIN + AM and SPS + AM; e, $p < 0.05$: different from SPS + Veh; f, $p < 0.01$: different from SPS + Veh and SPS + WIN + AM; g, $p < 0.05$: different from SPS + AM; h, $p < 0.05$: different from SPS + WIN; i, $p < 0.05$: different from SPS + WIN + AM and SPS + AM; j, $p < 0.01$: different from SPS + Veh).

latency between the days ($F_{(1,29)} = 4.39$; $p = 0.045$), with no significant interaction ($F_{(2,29)} = 1.35$; NS). One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(2,29)} = 4.57$; $p = 0.019$), Ext1 ($F_{(2,29)} = 3.32$; $p = 0.05$), and Ext3 ($F_{(2,29)} = 4.08$; $p = 0.027$). The Veh BLA group demonstrated decreased latency compared with the two other groups on Cond (SPS + Veh BLA: $p = 0.008$; SPS + WIN2 h BLA: $p = 0.041$) and Ext3 (SPS + Veh: $p = 0.05$; SPS + WIN2 h BLA $p = 0.011$). Also, the Veh BLA group demonstrated decreased latency compared with the SPS + Veh BLA group on Ext1 ($p = 0.016$) and Ext2 ($p = 0.041$). This suggests that WIN administered into the BLA 2 h post-SPS did not prevent the effects of stress on extinction.

Next, we examined whether microinjecting a low dose of the CB1 receptor antagonist AM251 (AM; 0.3 ng/0.5 μl) into the BLA would block the effects of WIN on SPS-induced alterations in IA conditioning and extinction. WIN and AM251 were administered into the BLA alone (SPS + WIN BLA and SPS + AM BLA) or co-administered in a single injection (SPS + WIN + AM BLA) 2 min after SPS (Figure 3c).

Mixed ANOVA (groups × days (6 × 4)) revealed a significant difference between the groups in terms of their latency to enter the dark side of the box ($F_{(5,63)} = 263.59$; $p = 0.0001$). There was a within-subject difference in the latency between the days ($F_{(1,63)} = 20.28$; $p = 0.0001$) and a significant interaction ($F_{(5,63)} = 3.03$; $p = 0.016$). One-way ANOVA applied on each day revealed that the significant main effect stemmed from a difference in latency between the groups on Cond ($F_{(5,63)} = 4.00$; $p = 0.003$), Ext1 ($F_{(5,63)} = 2.74$; $p = 0.027$), Ext2 ($F_{(5,63)} = 5.16$; $p = 0.0001$), and Ext3 ($F_{(5,63)} = 3.51$; $p = 0.007$). On Cond, the SPS + Veh

BLA group demonstrated increased latency compared with Veh BLA ($p = 0.001$), SPS + WIN + AM BLA ($p = 0.021$), SPS + AM BLA ($p = 0.020$), and NO SPS + AM BLA ($p = 0.002$). The Veh BLA group demonstrated decreased latency compared with the SPS + Veh BLA, SPS + WIN + AM BLA, and SPS + AM BLA groups on Ext1 ($p = 0.002$, $p = 0.027$, and $p = 0.049$, respectively), Ext2 ($p = 0.001$, $p = 0.004$, and $p = 0.011$, respectively), and Ext3 ($p = 0.003$, $p = 0.014$, and $p = 0.033$, respectively). Hence, SPS rats injected with vehicle, AM, or a combination of AM + WIN after SPS exposure showed increased fear retrieval (Ext1) and impaired extinction (Ext2–3). Importantly, the SPS + WIN + AM BLA group demonstrated increased latencies compared with the SPS + WIN BLA on Ext2 ($p = 0.030$), and Ext3 ($p = 0.038$), indicating that AM251 blocked the effects of WIN on extinction after SPS. The NO SPS + AM BLA group demonstrated decreased latency compared with the SPS + Veh BLA on Ext1 ($p = 0.016$), Ext2 ($p = 0.001$), and Ext3 ($p = 0.007$) and compared with the SPS + WIN + AM BLA and the SPS + AM BLA on Ext2 ($p = 0.004$ and $p = 0.01$, respectively), and Ext3 ($p = 0.021$ and $p = 0.042$, respectively), suggesting that AM by itself had no effect on extinction.

The Effects of SPS and WIN55,212-2 on ASR

Rats were exposed to SPS and then treated with vehicle or with WIN 2 min, 2, 24, or 48 h after SPS exposure. All rats were tested for their ASR levels 1 week after SPS (Figure 4a). One-way ANOVA revealed significant differences in mean ASR levels between the groups (all groups are presented in one graph; 2 min: $F_{(2,27)} = 10.79$, $p < 0.001$; 2 h: $F_{(2,26)} = 23.31$, $p < 0.001$; 24 h: $F_{(2,27)} = 7.88$, $p < 0.002$;
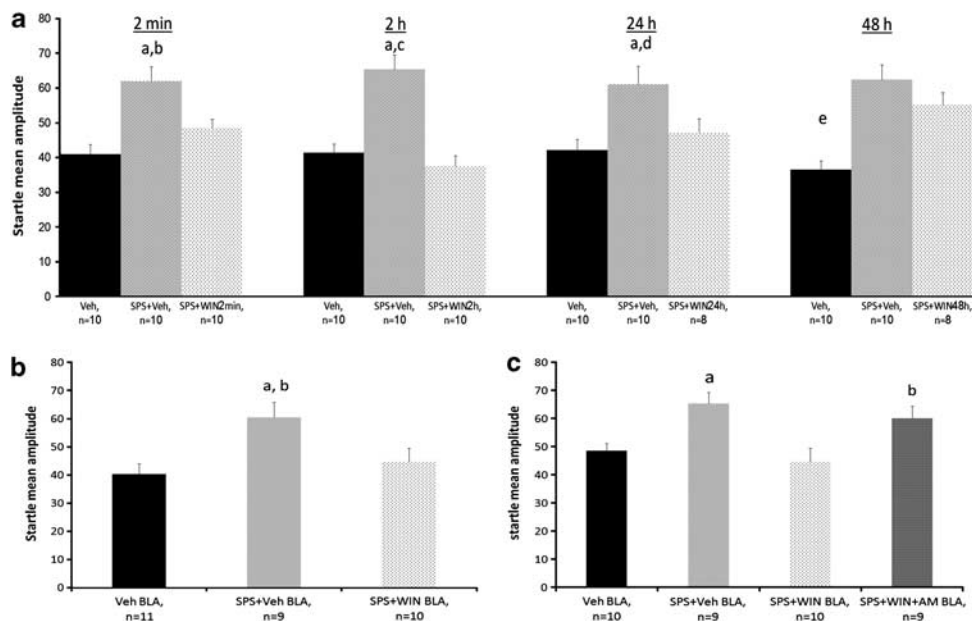

Figure 4 The effects of single-prolonged stress (SPS) exposure and WIN55,212-2 administration on acoustic startle response (ASR). (a) The SPS + Veh groups demonstrated a significantly enhanced ASR than the Veh groups and the SPS + WIN2 min, SPS + WIN2 h and SPS + WIN24 h groups (WIN: 0.5 mg/kg) (a, $p < 0.01$ compared with Veh; b, $p < 0.05$: compared with SPS + WIN2 min; c, $p < 0.01$: compared with SPS + WIN2 h; d, $p < 0.05$ compared with SPS + WIN24 h; e, $p < 0.01$: compared with SPS + Veh and SPS + WIN48 h). (b) WIN administered into the BLA (5 μg/side) 2 min after SPS exposure prevented the SPS-induced enhancement in ASR (a, $p < 0.01$: compared with Veh BLA; b, $p < 0.05$: compared with SPS + WIN BLA). (c) AM251 (0.3 ng/side), co-administered with WIN into the BLA after SPS exposure, blocked the effect of WIN on ASR (a, $p < 0.01$; b, $p < 0.05$: compared with Veh BLA and SPS + WIN BLA).

48 h: $F_{(2,27)} = 7.78$, $p < 0.002$). *Post hoc* comparison revealed that the SPS + Veh group demonstrated significantly potentiated ASR levels compared with the Vehicle groups ($p = 0.001$). Importantly, the SPS + Veh group demonstrated significantly potentiated ASR levels compared with the SPS + WIN2 min ($p = 0.011$), SPS + WIN2 h ($p = 0.001$), and SPS + WIN24 h ($p = 0.022$) groups, suggesting that WIN administered 2 min, 2 or 24 h post-SPS, but not 48 h post-SPS, prevented the enhancing effects of stress on ASR.

Injecting a higher dose of WIN (3 mg/kg) 2 h after SPS exposure also prevented the SPS-induced potentiation of ASR levels. One-way ANOVA revealed significant differences in mean ASR levels between the groups ($F_{(2,24)} = 7.5$, $p < 0.05$). *Post hoc* comparison revealed that the SPS + Veh group demonstrated significantly potentiated ASR levels (63.42 ± 2.81) compared with the Vehicle (40.91 ± 2.81, $p = 0.001$) and SPS + WIN2 h (49.48 ± 5.54) ($p < 0.05$) groups.

Next, we examined whether intra-BLA WIN would prevent the effects of SPS exposure on ASR potentiation. Rats were exposed to SPS and treated with vehicle or with WIN 2 min after SPS exposure. A vehicle group was used as control (Figure 4b). All rats were tested in the ASR paradigm 1 week after SPS. One-way ANOVA revealed significant differences in mean ASR levels between the groups ($F_{(2,30)} = 5.59$; $p < 0.009$). *Post hoc* comparison revealed that the SPS + Veh BLA group demonstrated significantly potentiated ASR levels compared with the Veh BLA ($p = 0.003$) and the SPS + WIN BLA ($p = 0.021$) groups.

Finally, to examine whether intra-BLA AM251 would block the effects of WIN on SPS-induced potentiation of ASR levels, rats were treated with Vehicle, or WIN, or AM251 in combination with WIN 2 min after SPS exposure (Figure 4c).

One-way ANOVA revealed significant differences in mean ASR levels between the groups ($F_{(3,37)} = 5.74$; $p < 0.003$). *Post hoc* comparison revealed that the SPS + Veh BLA group demonstrated significantly potentiated ASR levels compared with Veh BLA ($p = 0.006$) and SPS + WIN BLA ($p = 0.001$). The SPS + WIN + AM BLA group showed a significantly increased ASR compared with Veh BLA ($p = 0.05$) and SPS + WIN BLA ($p = 0.011$). Hence, AM251 microinjected into the BLA blocked the effects of WIN on ASR after SPS.

The Effects of SPS and WIN55,212-2 on HPA Axis Function

In our third experiment, we tested the effects of SPS on HPA axis function by measuring plasma CORT levels at rest and following the DST (Figure 5). CORT levels *at rest* were measured 2 min, 2, 24 h, or 1 week after SPS exposure and were compared with a control group. One-way ANOVA revealed significant differences in CORT levels between the groups ($F_{(4,31)} = 129.82$; $p < 0.001$; Figure 5a). *Post hoc* comparison revealed that the 2-min SPS and 2-h SPS groups demonstrated significantly increased CORT levels compared with the control group ($p = 0.001$ and $p = 0.005$,
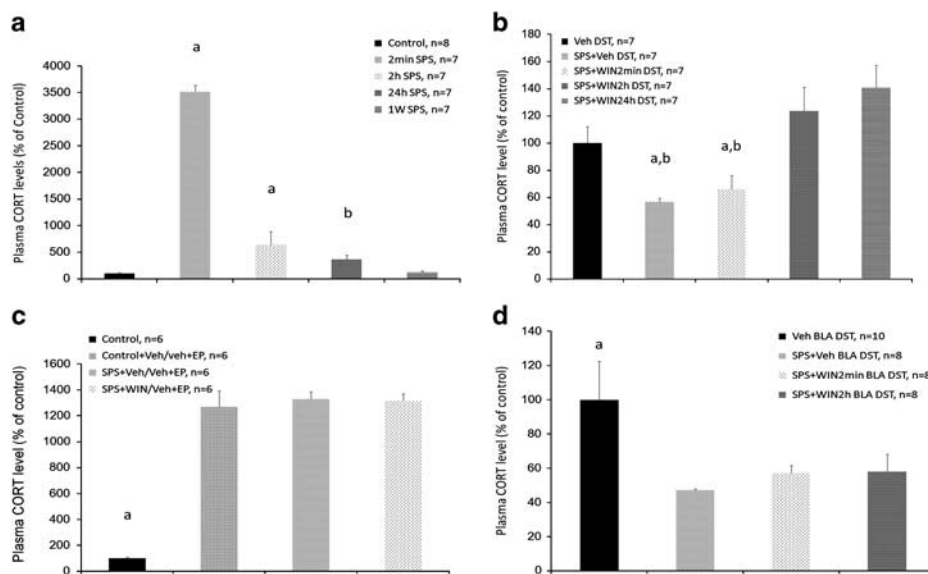

Figure 5 The effects of single-prolonged stress (SPS) and WIN55,212-2 administration on HPA axis function. (a) Rats decapitated 2 min, 2, or 24 h, but not 1 week (1W), after SPS exposure showed significantly increased levels of CORT compared with control rats. Data represent the mean values ± SEM expressed as a percentage of the CORT values of the control rats (132 ± 15 ng/ml) (a, $p < 0.01$; b, $p < 0.05$: compared with control). (b) SPS + Veh DST and SPS + WIN2 min DST groups show significantly enhanced negative feedback on the HPA axis as indicated by reduced CORT levels compared with the other groups following DEX injection and EP exposure (see Materials and methods). Data represent the mean values ± SEM expressed as a percentage of the CORT values of the Veh DST rats (46 ± 6 ng/ml) (a, $p < 0.05$: compared with Veh DST; b, $p < 0.01$: compared with SPS + WIN2 h DST and SPS + WIN24 h DST). (c) Control or SPS rats that were injected with vehicle or WIN and after 1 week treated with vehicle before EP exposure (no Dex) showed significantly increased levels of CORT compared with control rats (control; no exposure to EP or Dex). Data represent the mean values ± SEM expressed as a percentage of the CORT values of the control rats (116 ± 11 ng/ml) (a, $p < 0.01$: compared with all groups). (d) SPS rats injected with vehicle (SPS + Veh BLA DST) or WIN (SPS + WIN2 min BLA DST and SPS + WIN2 h BLA DST) into the BLA show significantly enhanced negative feedback on the HPA axis as indicated by reduced CORT levels compared with the control group (Veh BLA DST) following exposure to EP and DEX injection (see Materials and methods). Data represent the mean values ± SEM expressed as a percentage of the CORT values of the Veh rats (60 ± 13 ng/ml) (a, $p < 0.05$: compared with all groups).

respectively). Since the 24-h SPS group showed high CORT levels (367 ± 79) compared with the control group (100 ± 37), the two groups were compared using an independent samples t-test ($t_{(13)} = 4.30$, $p < 0.05$).

For the DST, rats were exposed to SPS and treated i.p. with vehicle or WIN 2 min, 2, or 24 h after SPS exposure. A vehicle group was used as control. One week after handling or SPS, Dex was administered before a second stress exposure, the elevated platform stress, and CORT levels were measured (Figure 5b). Note that in this figure, we are measuring CORT levels *in response to DST*. Reduced levels of CORT in response to DST indicate HPA axis inhibition. One-way ANOVA revealed significant differences in CORT levels between the groups in response to DST ($F_{(4,30)} = 8.19$; $p < 0.001$). *Post hoc* comparison revealed that the SPS + Veh DST group demonstrated significantly reduced CORT levels compared with the Veh DST ($p = 0.021$), SPS + WIN2 h DST ($p = 0.001$), and SPS + WIN24 h DST ($p = 0.0001$) groups. The SPS + WIN2 min DST group demonstrated significantly reduced CORT levels compared with the Veh DST ($p = 0.05$), SPS + WIN2 h DST ($p = 0.003$), and SPS + WIN24 h DST ($p = 0.001$) groups. Hence, the SPS group showed enhanced inhibition of the HPA axis in response to DST; WIN administered 2 or 24 h, but not 2 min, after SPS exposure prevented this enhanced inhibition.

Next, we demonstrated that SPS exposure by itself does not change the responsiveness to subsequent EP stress when no Dex is injected. Hence, control or SPS rats were injected with vehicle or WIN. After 1 week, rats were treated with vehicle before EP exposure (no Dex) and were compared with a control group (control; with no exposure to EP or Dex) (Figure 5c). One-way ANOVA revealed significant differences in CORT levels between the groups ($F_{(3,20)} = 69.017$; $p = 0.001$). *Post hoc* comparison revealed that the control group demonstrated significantly decreased CORT levels compared with all groups ($p < 0.001$). Hence, a similar response was observed in rats that were exposed to SPS and EP and rats that were exposed to EP without prior SPS exposure.

Finally, we examined whether intra-BLA WIN would prevent the effects of SPS exposure on HPA axis inhibition. Rats were exposed to SPS and microinjected into the BLA with vehicle or WIN 2 min or 2 h after SPS exposure. A vehicle group was used as control. One week after SPS, Dex was administered before a second stress exposure and CORT levels were measured (Figure 5d). One-way ANOVA revealed significant differences in CORT levels between the groups ($F_{(3,30)} = 2.96$; $p = 0.048$). *Post hoc* comparison revealed that the Veh BLA group demonstrated significantly increased CORT levels compared with SPS + Veh BLA DST ($p = 0.012$), SPS + WIN2 min BLA DST ($p = 0.038$), and SPS + WIN2 h BLA DST ($p = 0.042$). Hence, the SPS group showed enhanced inhibition of the HPA axis in response to DST and WIN administered into the BLA 2 min or 2 h after SPS exposure did not prevent this enhanced inhibition.

The Effects of SPS and WIN55,212-2 on Anxiety and Locomotion

We used the open field arena and the light-dark test to examine the effects of SPS and WIN (0.5 mg/kg) on anxiety and locomotion.

Table 1 The Effects of Single-Prolonged Stress (SPS) Exposure and WIN55,212-2 Administration on Locomotion and Anxiety in the Open-Field Test

	Time in center (s)	Distance covered (cm)
Veh ($n = 14$)	6.15 ± 1.56*	1350 ± 101.77
SPS+Veh ($n = 7$)	0.90 ± 0.36	1314.28 ± 162.45
SPS+WIN 2 min ($n = 7$)	1.36 ± 0.50	1550 ± 48.79
SPS+WIN 2 h ($n = 7$)	2.23 ± 0.65	1482.85 ± 100.99
SPS+WIN 24 h ($n = 8$)	2.86 ± 0.73	1221.37 ± 220.36
SPS+WIN 48 h ($n = 7$)	1.21 ± 1.21	1300 ± 124.4

The Veh group spent more time in the center of an open field compared with all other groups. *$p < 0.05$.

Table 2 The Effects of Single-Prolonged Stress (SPS) Exposure and WIN55,212-2 Administration on Anxiety in the Light-dark Test

	Time in light side (s)	Entries to light side
Veh ($n = 9$)	39.97 ± 8.36*	2.55 ± 046
SPS+Veh ($n = 8$)	7.28 ± 2.68	1.28 ± 0.45
SPS+WIN 2 min ($n = 8$)	19.06 ± 7.86	1.7 ± 0.52
SPS+WIN 2 h ($n = 8$)	13.23 ± 9.57	1.28 ± 0.56

The Veh group spent more time in the light side in the light-dark test compared with all other groups. *$p < 0.05$.

Rats were tested in the open field 1 week after SPS (Table 1). One-way ANOVA revealed significant differences in time in center between the groups ($F_{(5,44)} = 3.33$; $p < 0.05$). *Post hoc* comparison revealed that the Veh group spent significantly more time in the center than all groups ($p < 0.05$). As for the distance covered, one-way ANOVA did not reveal a significant difference between the groups ($F_{(5,44)} < 1$; NS), suggesting that neither SPS nor WIN administration affected gross motoric behavior.

A similar effect was found in the light-dark test conducted 1 week after SPS (Table 2). One-way ANOVA revealed significant differences in time in the light side between the groups ($F_{(3,29)} = 5.04$; $p < 0.05$). *Post hoc* comparison revealed that the Vehicle group spent significantly more time in the light side than all groups ($p < 0.05$).

DISCUSSION

The main findings of the current study are that exogenous systemic or intra-BLA administration of the CB1/CB2 cannabinoid receptor agonist WIN55,212-2 normalizes behavioral and neuroendocrine abnormalities resulting from prior stress exposure in a rat model of PTSD. We also demonstrate that there may be an optimal time window for treatment with cannabinoids after exposure to a highly stressful event and that CB1 receptors in the BLA contribute to some of the preventive effects of WIN.

Cannabinoid Receptor Activation Prevents the Effects of Stress on IA Extinction and ASR

WIN administered systemically 2 min, 2, or 24 h after SPS exposure prevented the stress-induced disruption of extinction learning and enhancement of ASR. When WIN was injected 48 h after trauma exposure, it was too late to reverse the effects of stress, and in fact resulted in the impairment of extinction. These findings suggest that there may be an optimal time window for pharmacological intervention following trauma exposure.

Importantly, WIN administered *into the BLA* 2 min after SPS also reversed the stress-induced effects on IA and ASR. This suggests the possible involvement of cannabinoid receptors in the BLA in preventing the effects of stress on IA and ASR, when the activation takes place in close proximity to stress exposure. This is consistent with studies suggesting that cannabinoids in the amygdala serve to attenuate neuronal and behavioral responses to aversive environmental stimuli (Patel *et al*, 2005; Ganon-Elazar and Akirav, 2009). The different time table for the systemic and intra-BLA effects of WIN may suggest that there are other brain areas besides the BLA that are involved in the preventive effect of WIN on SPS-induced symptoms (eg, hippocampus and prefrontal cortex).

When a low and non-impairing dose of the CB1 receptor antagonist AM251 was co-administered with WIN into the BLA after SPS, it blocked the effects of WIN on IA and ASR, suggesting that the preventive effects of WIN are mediated via an activation of CB1 receptors in the BLA. Yet, we cannot completely rule out the possibility that other targets of WIN (eg, CB2 receptors) contributed to its effects.

Systemic Cannabinoid Receptor Activation Prevents the Effects of Stress on HPA axis Function

Rats exposed to SPS showed enhanced inhibition of the HPA axis as indicated by reduced CORT levels in response to the DST, corroborating previous studies (Liberzon *et al*, 1997; Kohda *et al*, 2007). Importantly, WIN administered systemically 2 or 24 h, but not 2 min, after SPS exposure prevented the enhancement of HPA axis inhibition. However, WIN microinjected into the BLA 2 min or 2 h after SPS exposure did not prevent the stress-induced alterations to HPA axis function. The DST assesses CORT levels following negative feedback to the pituitary to suppress the secretion of adrenocorticotropic hormone. Thus, activating cannabinoid receptors in the BLA may not affect the DST-induced HPA axis inhibition.

Several studies have shown that activating CB1 receptors or increasing *N*-arachidonylethanolamine (AEA) signaling prevents some of the effects of stress in the amygdala and hippocampus and can reduce stress-induced HPA axis activation (Ganon-Elazar and Akirav, 2009; Patel *et al*, 2004; Gorzalka *et al*, 2008). On the other hand, exposure to stress causes significant reductions in amygdalar and hippocampal AEA content (Patel *et al*, 2005a; Hill *et al*, 2005; Rademacher *et al*, 2008; Gorzalka *et al*, 2008). Hence, there are probably non-amygdala brain areas that are also involved in suppressing stress-induced behaviors and reducing HPA axis output following cannabinoids enhancement (eg, the hippocampus). Specifically, the finding that

AEA content is reduced within the amygdala at the termination of stress exposure (Hill *et al*, 2009) may support a temporary role for cannabinoid receptor activation in the BLA in modulating the stress response.

Interestingly, resting CORT levels measured at different time points following SPS exposure were extremely high immediately after the trauma and remained significantly high up to 24 h thereafter. We have previously shown that intra-BLA WIN reversed the stress-induced increase in CORT levels (Ganon-elazar and Akirav, 2009). Hence, it is possible that the preventive effects of cannabinoids within a time window ranging from 2 min to 24 h after SPS are associated with their inhibition of the elevated CORT levels that follow stress exposure.

The Effects of Cannabinoid Receptor Activation on SPS-Induced Anxiety

WIN administration did not prevent the SPS-induced enhancement of anxiety as measured in the open field and dark-light tests 1 week after trauma. This lack of effect is particularly interesting since it may indicate that cannabinoid receptor activation reverses the effects of SPS on PTSD-like behavioral and neuroendocrine measures (ie, disrupted extinction, enhanced ASR, and HPA inhibition), but that it is not necessarily effective in blocking all the effects of stress exposure.

Importantly, this result suggests that the effects of WIN in preventing PTSD-like symptoms are not due to a general 'relaxation effect' or to an erasure of the stressful event, since rats injected with WIN after SPS still exhibit unconditioned anxiety. Hence, rats that were injected with WIN after SPS are still anxious, but do not demonstrate the two main PTSD-like symptoms (ie, enhanced ASR and HPA-negative feedback).

The Involvement of Cannabinoid Receptor Activation in the BLA in Preventing the Effects of SPS on Behavioral and Neuroendocrine Measures

Our findings suggest that at least some of the beneficial effects of cannabinoids administered following trauma exposure are mediated by the BLA. Patel *et al* (2004) found a synergistic interaction between environmental stress and CB1 receptor activation in the amygdala by demonstrating robust Fos induction within the BLA and the central amygdala following restraint stress and CB1 agonist administration. Hill *et al* (2009) have recently shown that the content of AEA in the BLA is reduced at the termination of restraint stress (also see Patel *et al*, 2005a and Rademacher *et al*, 2008) and that AEA content is negatively correlated with the magnitude of the CORT response to stress. Inhibiting fatty acid amide hydrolase (an endocannabinoid-deactivating enzyme) activity within the BLA reduces the CORT response to stress, suggesting that AEA content in the BLA has a function in constraining HPA axis activation. Together with our findings that intra-BLA WIN reduces the CORT response to stress (Ganon-Elazar and Akirav, 2009), these studies suggest cannabinoid modulation in the BLA of HPA axis activation in proximity to stress exposure. This could explain why WIN injected into the

BLA after stress termination (ie, 2 min, but not 2 h, after SPS exposure) blocked the stress-induced effects on behavior.

The cannulae were implanted into the BLA and although we used a small volume of infusion (0.5 μl), the possibility of injection spread to other structures, especially the central amygdala nucleus, cannot be completely ruled out. Yet, CB1 receptors are expressed at high levels in the BLA (Herkenham *et al*, 1990; Katona *et al*, 2001; Tsou *et al*, 1997) but their expression in the central amygdala is less clear (Katona *et al*, 2001; Tsou *et al*, 1997; Cota *et al*, 2007; but see Kamprath *et al*, 2011).

Conclusions

Our findings are of considerable interest since they indicate a relatively broad therapeutic time window in the aftermath of trauma exposure for preventive treatment with CB1 agonists. Although the precise mechanism by which cannabinoid receptor activation prevents the stress-induced behavioral and neuroendocrine modifications remains to be clarified, our findings suggest a crucial contribution of CB1 receptors in the BLA.

Furthermore, the results extend previous findings to another stress model and to a post-trauma treatment configuration that are more relevant to clinical context and add to the growing body of data pointing to a therapeutic potential of cannabinoids for treatment of PTSD.

DISCLOSURE

The authors declare no conflict of interest.

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