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Using c-Jun to identify fear extinction learning-specific patterns of neural activity that are affected by single prolonged stress

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Abstract

Neural circuits via which stress leads to disruptions in fear extinction is often explored in animal stress models. Using the single prolonged stress (SPS) model of post traumatic stress disorder and the immediate early gene (IEG) c-Fos as a measure of neural activity, we previously identified patterns of neural activity through which SPS disrupts extinction retention. However, none of these stress effects were specific to fear or extinction learning and memory. C-Jun is another IEG that is sometimes regulated in a different manner to c-Fos and could be used to identify emotional learning/memory specific patterns of neural activity that are sensitive to SPS. Animals were either fear conditioned (CS-fear) or presented with CSs only (CS-only) then subjected to extinction training and testing. C-Jun was then assayed within neural substrates critical for extinction memory. Inhibited c-Jun levels in the hippocampus (Hipp) and enhanced functional connectivity between the ventromedial prefrontal cortex (vmPFC) and basolateral amygdala (BLA) during extinction training was disrupted by SPS in the CS-fear group only. As a result, these effects were specific to emotional learning/memory. SPS also disrupted inhibited Hipp c-Jun levels, enhanced BLA c-Jun levels, and altered functional connectivity among the vmPFC, BLA, and Hipp during extinction testing in SPS rats in the CS-fear and CS-only groups. As a result, these effects were not specific to emotional learning/memory. Our findings suggest that SPS disrupts neural activity specific to extinction memory, but may also disrupt the retention of fear extinction by mechanisms that do not involve emotional learning/memory.

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FINANCIAL DISCLOSURE

Dr. Dayan Knox, Briana Stanfield, Jennifer Staib, Dr. Nina David, Thomas DePietro, Marisa Chamness, Elizabeth Schneider, Samantha Keller, and Caroline Lawless have no conflict of interest concerning the findings presented in this manuscript.

Keywords

PTSD; fear; anxiety; extinction; c-Jun

1.1 INTRODUCTION

Fear extinction refers to the process of inhibiting fear expression by repeated non-reinforced exposure to a feared stimulus [1–6]. The neurobiology of fear extinction is often explored in the Pavlovian fear conditioning paradigm. In this paradigm, an innocuous stimulus such as a tone (i.e. conditioned stimulus (CS)) is paired with an unconditioned stimulus (UCS) such as a footshock. Animals show freezing to the CS after fear conditioning, but after repeated non-reinforced CS presentations, conditioned freezing decreases (i.e. extinction training). The effectiveness of extinction training in inhibiting conditioned freezing is then examined by presenting the extinguished CS during a separate extinction test (i.e. extinction retention).

Extinction retention requires neural plasticity in the infralimbic cortex (IL), basolateral amygdala (BLA), and ventral hippocampus (vHipp) [7–12]. IL input to the intercalated cell masses of the amygdala (ITC) is critical for inhibiting conditioned fear expression [13, 14]. The prelimbic cortex (PL) is critical for conditioned fear expression [15] and vHipp input to the PL is critical for regulating expression of conditioned fear [16, 17]. The dorsal hippocampus (dHipp) is critical for contextual modulation of fear extinction [2, 17–20]. Thus, a neural circuit (referred to here as the fear extinction circuit) comprised of the PL, IL, dHipp, BLA, and vHipp is critical for extinction retention.

Extinction retention is critical for emotional regulation and is disrupted in stress-induced anxiety disorders such as post traumatic stress disorder (PTSD) [1, 3, 21, 22]. Using animal models, previous studies have identified neural circuits through which stress exposure leads to extinction retention deficits. If the stress of fear conditioning carries over to extinction training, extinction retention is disrupted [23]. This effect may be driven by changes in spontaneous and CS-evoked single unit activity in the IL during extinction training and testing [24]. Chronic stress leads to deficits in extinction memory [25–28], possibly due to stress-induced changes in IL single unit activity during extinction testing [29].

Another approach used to map neural circuits critical for mediating stress-induced extinction retention deficits is expression of immediate early genes (IEGs). c-Fos, a dimer of the rapidly regulated transcription factor AP-1 [30–32], is often used to measure neural activity across a wide range of brain regions [33]. Using this methodology a previous study has observed that chronic stress may disrupt extinction retention by enhancing neural activity in the dHipp and BLA during extinction testing [34]. Single prolonged stress (SPS) refers to serial exposure to restraint, forced swim, and ether and consistently results in extinction retention deficits [35–38]. A recent study we conducted observed that enhancements in IL c-Fos levels and inhibition of BLA c-Fos levels during extinction training are disrupted in SPS rats [39]. Furthermore, inhibition of vHipp and BLA c-Fos levels is disrupted during extinction testing in SPS rats, while functional connectivity between the vHipp and BLA is enhanced in SPS rats [39]. However, these effects were also observed in animals that were presented with auditory tones in the absence of footshocks, then subjected to extinction

training and testing protocols. This suggests that these stress-induced effects were not specific to fear extinction learning and memory as they occurred in SPS animals that that never learned fear and so could not have learned extinction. C-Fos is a subunit of AP-1 and subunits of AP-1 are not always regulated with neural stimulation in an identical manner [30, 31, 40–42]. This raises the possibility that using other dimers of AP-1 to map neural activity across the fear extinction circuit could reveal learning-specific patterns of neural activity that are sensitive to SPS.

In this study, a set of SPS and control rats were fear conditioned, then subjected to extinction training and testing (CS-fear). A second set of SPS and control rats were presented with CSs only instead of fear conditioning, then presented with tones again in an identical manner to animals subjected to extinction training and testing (CS-only). Animals in the CS-fear group formed fear memory then extinction memory, whereas animals in the CS-only group were repeatedly presented with the same CS and never learned fear or extinction. After extinction training or testing, subsets of rats were euthanized to assay c-Jun levels in the fear extinction circuit. We also assayed c-Jun levels in a third set of SPS and control rats after immediate removal from the housing colony to establish baseline c-Jun levels. This experimental design allowed us to characterize patterns of c-Jun expression that were specific to emotional learning/memory (e.g. extinction memory) vs. changes in c-Jun that occurred due to repeated tone presentation (e.g. habituation-like processes). The experimental design is illustrated in (Figure 1).

1.2 METHODS AND MATERIALS

1.2.1 Animals

One hundred and one adult male Sprague Dawley rats (150 g upon arrival), obtained from Charles River were used in this study. Upon arrival all rats were pair housed for 3–5 days. All rats had ad libitum access to food during this time period, but were then kept on a diet of 23g of rat chow per day, which is the manufacturer's recommended diet (LabDiet St. Louis MO). Throughout the study, rats had ad libitum access to water. Experimental manipulations commenced after rats had been in the housing colony for at least five days. Rats were on a 12 hour light/dark cycle. All experimental procedures were performed in the animals' light cycle and all behavioral tests were conducted between 9:00 am and 2:00 pm. All experiments were approved by the University of Delaware Institutional Animal Care and Use Committee following guidelines established by the NIH.

1.2.2 Experimental design, SPS, and behavioral procedures

All rats were initially assigned to the SPS or control stress group. SPS was conducted as previously described [43, 44]. SPS consisted of two hours of restraint, 20 minutes of forced swim, and ether exposure until general anesthesia was induced. Rats assigned to the control group were placed in a novel room for the duration of SPS. After SPS, all rats were returned to the housing colony and a post-stress incubation period of seven days elapsed prior to behavioral testing, because this period is needed to observe behavioral and physiological effects in the SPS model [35, 44–46].

All SPS and control rats were then divided into two groups. One group of SPS and control rats were removed from the housing colony and immediately euthanized. We used this group of rats to establish baseline c-Jun levels in the fear extinction circuit. The other group of SPS and control rats were further subdivided into two groups. One group was subjected to fear conditioning, then extinction training and testing. We refer to this group as CS-fear. The other group of rats were presented with CSs in the absence of footshock during fear conditioning, then subjected to extinction training and testing. We refer to this group as CS-only.

Fear conditioning and extinction sessions were conducted as previously described [35, 47, 48]. A 10s auditory CS (2 kHz, 80 dB) co-terminated with a 1mA, 1s footshock UCS in a distinct context (Context A). The CS-only group had CS presentations in the absence of footshocks. Extinction training commenced one day after fear conditioning and occurred in Context B with 30 CS presentations. Extinction testing commenced one day after extinction training in Context B and consisted of 10 CS presentations. Contexts A and B were created by manipulating multiple sensory cues [35]. All behavioral sessions consisted of a baseline period of 210 s and inter-stimulus intervals (ISIs) of 60 s. All behavior was recorded and scored at a later time as described below.

1.2.3 c-Jun immunocytochemistry

Rats were euthanized via rapid decapitation either after immediate removal from the housing colony or 60 minutes after the start of extinction training or testing. Brains were then extracted and flash frozen in chilled isopentane and stored in a -80°C freezer until further processing. Brains were then thawed to -13°C in a cryostat (Leica CM1350) and 30 μm sections through the vmPFC (AP: 3.72mm – 2.52mm), dHipp (AP: -2.4mm – -4.2mm , BLA (AP: -2.4mm – -3.36mm), and vHipp (AP: -4.92mm – -5.88mm) were mounted onto superfrost slides. All coordinates were based on the atlas of Paxinos and Watson [49]. Brain sections were then stored in a -80°C freezer until time of assay.

In order to perform c-Jun immunocytochemistry, sections were fixed in 4% paraformaldehyde in .2M phosphate buffered saline (PBS). Sections were then incubated in Triton X-100, rinsed in .1M tris buffered saline (TBS) and incubated in 3% goat serum. Sections were rinsed again in TBS and incubated with a rabbit polyclonal c-Jun antibody (Santa Cruz Biotechnology, Santa Cruz CA, sc-52) at a dilution of 1:1000 in PBS overnight at 4°C . Sections were then rinsed in TBS containing 0.01% Tween-20 (TBS-T). After this, sections were incubated in a solution consisting of TBS, 1.5% goat serum, 0.1% Triton X-100, and goat anti-rabbit IgG antibody 800CW (Li-cor Biotechnology 926-32211) in a dilution of 1:2000 for two hours. Sections were rinsed in TBS-T, TBS, and then deionized water. Section were then left to air dry overnight.

1.2.4 Data analysis and statistical analysis

The behavioral data for these animals have been published elsewhere [39], and are thus not presented in this manuscript. C-Jun assays were scored by individuals blind to group assignments of animals and/or rationale for the study. Data analyses were checked by two or more individuals. In every c-Jun assay, representative animals from every independent group

in this study (baseline, CS-fear, CS-only, SPS, Control) were included in each assay. The secondary antibody used in the c-Jun immunocytochemistry procedure fluoresces at 780nm (i.e. near infrared fluorescence) [for example see 48]. This activity was used to measure c-Jun levels in specific brain regions. Dried brain sections, treated for c-Jun immunocytochemistry, were scanned at 21 μ m resolution in the Li-cor Odyssey CLx scanner. Fluorescent activity in a particular brain region (8 – 24 samples per brain region) was then expressed as a percent change from activity in the corpus callosum. We referred to this as signal activity.

Signal activity from all brain regions in the baseline condition was subjected to t-test (SPS vs. control). Signal activity obtained from rats euthanized after extinction training and testing was normalized with respect to baseline signal activity. For example, IL signal activity from a SPS rat in the CS-only condition was normalized relative to averaged baseline IL signal activity of SPS rats. We refer to this as normalized activity. Normalized activity was constructed so that signal activity during extinction training and testing that was equal to baseline signal activity would yield a normalized score of 100% (i.e. (signal activity / averaged baseline activity) $\times$ 100). Normalized activity in the fear extinction circuit (i.e. IL, PL, dHipp (dCA1, dCA3, and dDG), BLA (lateral (LA) and basal (BA) regions separately), and vHipp (vCA, vDG)) during extinction training and testing was subjected to a stress (SPS vs. control) $\times$ learning treatment (CS-only vs. CS-fear) factor design. For all factor designs main and simple effects were analyzed using analysis of variance (ANOVA) while main and simple comparisons were analyzed using t-test with Bonferroni corrections applied where necessary. We also employed one sample t-tests to determine if a specific group mean was different from 100%. If multiple one-sample t-tests were performed for normalized activity, then Bonferroni corrections were also applied to these tests. All graphs plot means along with standard error of the means.

In order to examine functional connectivity within the fear extinction circuit, we applied factor analyses to the c-Jun dataset using similar methodology to that previously described [39]. Factor analysis, when applied to a set of variables, identifies correlations among variables and groups these variables as a single factor, with the strength of the correlation determining whether variables are grouped into a factor [50]. As a result, factor analysis applied to our c-Jun dataset can be used to identify patterns of correlated neural activity (i.e. functional connectivity) among components of the fear extinction circuit. We applied factor analysis to signal activity in the IL, PL, dCA1, dCA3, dDG, LA, BA, vCA and vDG of SPS and control rats. This was done separately in all treatments. Factors were extracted using principal component analysis. Only factors with eigenvalues greater than 1 were considered (Kaiser Rule) and the entire factor analysis was only considered valid if it accounted for greater than 75% of the variance within a dataset. For a variable to be considered as part of a factor, correlation of that variable with a factor had to be greater than .5. If a variable was part of two factors and had correlation values greater than .5, the higher correlation value was used to group the factor. Thus, a variable could not be part of two different factors in this analysis. Lastly, we primarily interpreted patterns of functional connectivity within circuits that have been previously shown to be critical for inhibition of fear memory or extinction memory formation. All statistical analyses were performed in IBM SPSS Statistics package 4.

1.3 RESULTS

1.3.1 c-Jun immunoreactivity

Representative c-Jun stained brain sections are illustrated in Figure 2. We have previously validated this method of detecting the protein products of IEGs in brain tissue using immunocytochemistry [39] and separately demonstrated that signal activity observed in c-Jun assays was due to detection of c-Jun and not autofluorescence or non-specific secondary antibody binding (Supplemental data).

1.3.1.1 vmPFC—Baseline PL c-Jun levels were equivalent between SPS and control rats [$t_{(27)} = .909$, $p = .371$]. Neither stress (SPS vs. control) nor learning treatment (CS-fear vs. CS-only) affected PL c-Jun levels during extinction training ($ps > .05$). There was a significant increase in PL c-Jun levels during extinction training in all rats. This effect was revealed by a significant one-sample t-test for PL c-Jun levels collapsed across all groups [$t_{(35)} = 2.949$, $p = .006$]. During extinction testing there were no stress or learning treatment effects ($ps > .05$). There was a significant increase in PL c-Jun levels [one-sample t-test collapsed across groups: $t_{(33)} = 3.371$, $p = .002$]. These results are illustrated in Figure 3A.

Baseline IL c-Jun levels were equivalent between SPS and control rats [$t_{(27)} = .903$, $p = .375$]. Neither SPS nor learning treatment affected IL c-Jun levels during extinction training ($ps > .05$), but there was a significant increase in IL c-Jun levels during extinction training in all rats [$t_{(35)} = 2.447$, $p = .02$]. Neither stress nor learning treatment affected IL c-Jun levels during extinction testing ($ps > .05$). However, IL c-Jun levels were enhanced in all rats [$t_{(35)} = 2.414$, $p = .021$]. These results are illustrated in Figure 3A.

1.3.1.2 dHipp—Baseline dHipp c-Jun levels were equivalent between SPS and control rats [dCA1: $t_{(27)} = 1.21$, $p = .237$; dCA3: $t_{(27)} = .867$, $p = .394$; dDG: $t_{(27)} = 1.168$, $p = .154$]. Neither stress nor learning treatment had any effects on c-Jun levels in the dCA1 ($ps > .05$), but c-Jun dCA1 levels were attenuated during extinction training [$t_{(35)} = -2.8$, $p = .008$]. SPS rats in the CS-fear treatment had enhanced c-Jun levels in dCA3 and dDG during extinction training relative to the other treatments. These effects were revealed by significant stress $\times$ treatment interactions [dCA3 – $F_{(1,32)} = 4.245$, $p = .048$; dDG – $F_{(1,32)} = 5.587$, $p = .022$]. However, c-Jun levels in the SPS/CS-fear treatment were not statistically different to baseline [dCA3 – $t_{(8)} = .185$, $p = .858$; dDG – $t_{(8)} = 1.3$, $p = .23$]. Furthermore, c-Jun levels were below baseline in the Control/CS-only [dCA3 – $t_{(8)} = -6.851$, $p < .001$; dDG – $t_{(8)} = -3.256$, $p = .048$], Control/CS-fear [dCA3 – $t_{(8)} = -8.644$, $p < .001$; dDG – $t_{(8)} = -7.161$, $p < .001$], and SPS/CS-only [dCA3 – $t_{(8)} = -4.276$, $p = .012$; dDG – $t_{(8)} = -3.676$, $p = .024$] groups. Thus, the SPS/CS-fear treatment disrupted inhibited c-Jun levels in the dCA3 and dDG during extinction training.

There were no effects of stress or learning treatment ($ps > .05$) for dCA1 and dCA3 c-Jun levels during extinction testing. C-Jun levels in dCA3 were attenuated relative to baseline in all rats [$t_{(35)} = -4.111$, $p < .001$]. SPS enhanced dDG c-Jun levels [main effect of stress: $F_{(1,32)} = 6.028$, $p = .02$] during extinction testing relative to controls. C-Jun levels were also attenuated relative to baseline in all SPS [$t_{(35)} = -5.439$, $p < .001$] and control [$t_{(35)} = -5.439$, $p < .001$] rats, which suggests inhibition of dDG c-Jun levels were attenuated in SPS

rats in both the CS-fear and CS-only treatments during extinction testing. These results are illustrated in Figure 3B.

1.3.1.3 BLA—Baseline c-Jun levels in the LA [$t_{(26)} = 1.428$, $p = .165$] and BA [$t_{(26)} = 1.431$, $p = .164$] were equivalent between SPS and control rats. There were no effects of stress or learning treatment for c-Jun LA and BA levels during extinction training ($ps > .05$). Inhibited LA [$t_{(35)} = -3.03$, $p = .005$], but not BA [$t_{(35)} = -.858$, $p = .397$], c-Jun levels were observed during extinction training in all rats. During extinction testing, enhanced LA [main effect of stress: $F_{(1,32)} = 6.28$, $p = .017$] and BA [main effect of stress: $F_{(1,32)} = 6.315$, $p = .017$] c-Jun levels in SPS rats within the CS-fear and CS-only treatments were observed. These results are illustrated in Figure 3C.

1.3.1.4 vHipp—Baseline vHipp c-Jun levels were equivalent between SPS and control rats [vCA: $t_{(25)} = .005$, $p = .996$; vDG: $t_{(25)} = .55$, $p = .587$]. Neither stress nor learning treatment had any effects on c-Jun levels in the vHipp during extinction training ($ps > .05$), but c-Jun levels in all regions of the vHipp were significantly decreased from baseline [vCA - $t_{(34)} = -3.563$, $p = .001$; vDG - $t_{(34)} = -3.973$, $p < .001$]. Neither stress nor learning treatment had any effects on vHipp c-Jun levels during extinction testing ($ps > .05$). Similar to what was observed with extinction training, c-Jun levels in all vHipp regions were significantly decreased from baseline [vCA - $t_{(35)} = -9.759$, $p < .001$; vDG - $t_{(35)} = -9.718$, $p < .001$]. These results are illustrated in Figure 3D.

1.3.2 Factor Analysis

Factor analyses were used to identify functional connectivity within the fear extinction circuit that were altered by learning treatment and stress factors. The results of these analyses are illustrated in Figure 4. In each factor analysis, the rotated component matrix, variance explained by the factor analysis, cartoon of factors in brain sections, and schematic of factors are illustrated. For control rats in the CS-fear treatment, functional connectivity patterns at baseline and during extinction testing were the same. During extinction training, there was enhanced selective functional connectivity between the vmPFC and BLA (vmPFC $\longleftrightarrow$ BLA). In SPS rats, functional connectivity patterns during baseline and extinction testing were different in the CS-fear treatment. During extinction training, there was no vmPFC $\longleftrightarrow$ BLA functional connectivity in SPS rats in the CS-fear treatment. Enhanced vmPFC $\longleftrightarrow$ vHipp and BLA $\longleftrightarrow$ dHipp functional connectivity in all SPS rats (i.e. CS-fear and CS-only) was observed during extinction testing.

1.4 DISCUSSION

In this study we identified patterns of neural activity during extinction training that were disrupted by SPS and specific to emotional learning/memory. There was selective vmPFC $\longleftrightarrow$ BLA functional connectivity during extinction training in control rats in the CS-fear treatment, and this effect was not observed in SPS/CS-fear rats. Both the vmPFC and BLA have been implicated in extinction memory formation (see 1.1 Introduction), with increased vmPFC control of BLA neural activity specifically being implicated in extinction memory [1, 13, 51]. These findings suggests that SPS-induced disruptions of

vmPFC $\longleftrightarrow$ BLA functional connectivity during extinction training weakens extinction memory and leads to deficits in extinction retention.

Inhibited dDG and dCA3 c-Jun levels during extinction training in control rats were disrupted in the SPS/CS-fear rats only. Given the role of the dHipp in contextual learning during emotional events [2, 17–20], these findings suggest a stress-induced change in neural activity that is specific to contextual learning/memory during extinction training. How might this effect lead to extinction retention deficits? SPS increases NMDA receptor mRNA expression [37] and enhances Akt signaling in the Hipp [52]. Both activation of NMDARs and enhanced Akt signaling in the Hipp have been implicated in contextual memory [53–57]. Differences in contextual features between extinction training and testing can prime fear memory retrieval during extinction testing [2, 18, 58, 59]. If failure to inhibit neural activity during extinction training in the dDG and dCA3 of SPS rats leads to enhanced contextual memory formation for the extinction training context, this may increase the sensitivity of SPS rats to contextual inconsistencies (temporal in nature since extinction training and testing were conducted in the same physical context) between extinction training and testing, which would prime cued fear memory retrieval. While speculative previous studies have shown that SPS enhances contextual fear memory formation [38, 60, 61]. SPS also enhances cued fear memory retrieval during extinction testing when driven by a context shift between extinction training and testing [35]. Thus, extinction retention deficits in the SPS model could emerge due to enhanced sensitivity to contextual features between extinction training and testing. Furthermore, enhanced sensitivity to these differences could be driven, in part, by disinhibited neural activity in dDG and dCA3 during extinction training in SPS rats. It should be noted that SPS disrupts spatial learning and memory in the Morris Water Maze [62, 63]; a task that is also dependent on hippocampal function [64]. These findings suggest that the effects of SPS on contextual learning during emotional events (e.g. fear, extinction) and contextual processing during spatial navigation are different. It is unclear why this would be, but the divergent effects of SPS on two tasks that involve contextual processing and are hippocampal dependent point to the need to further examine the effect of SPS on hippocampal function as it relates to contextual learning and memory.

Similar to what we previously observed [35], there were patterns of neural activity that were disrupted by SPS in a fear extinction learning/memory non-specific manner as they were observed in SPS rats in the CS-fear and CS-only treatments. During extinction testing, there was enhanced c-Jun expression in the BLA, a failure to inhibit dDG c-Jun levels, enhanced vmPFC $\longleftrightarrow$ vHipp functional connectivity, and enhanced BLA $\longleftrightarrow$ dHipp functional connectivity. vHipp connectivity to the vmPFC (specifically PL) is critical for gating expression of conditioned fear and enhancing conditioned fear when extinction is tested in a context inconsistent with the extinction context [16, 17]. The dHipp is critical for contextual control of extinction memory retrieval [18, 19]. The observation that these effects are observed in SPS rats that never learned fear (and thus never learned fear extinction), suggest that SPS effects on neural activity in certain neural substrates are not limited to fear and/or extinction memory phenomena, but may be observed in other behavioral phenomena that are dependent on these neural substrates (e.g. appetitive conditioning, social bonding [65, 66]). Further research examining these possibilities is needed.

1.4.1 Mechanisms by which SPS disrupts extinction retention

Through what mechanisms might SPS lead to the changes in neural activity (measured using c-Jun) and functional connectivity observed in this study? Previous studies have demonstrated that SPS disrupts neural function in the vmPFC and BLA. SPS decreases excitatory tone and enhances apoptosis in the vmPFC [43, 67]. Enhanced vmPFC apoptosis induced by SPS may be due to SPS-induced changes in signaling pathways in the endoplasmic reticulum that are critical for inhibiting apoptosis [67, 68]. SPS also enhances apoptosis in the BLA [69, 70]. Thus, SPS-induced changes in excitatory tone in the vmPFC and apoptosis in the vmPFC and BLA could lead to disruptions in vmPFC $\longleftrightarrow$ BLA during extinction training and enhancements in vmPFC $\longleftrightarrow$ vHipp during extinction testing, though more research is needed to better delineate how this may occur.

During extinction testing there was enhanced BLA neural activity in SPS rats. SPS-induced changes in molecular signaling at the CB1 receptor in the BLA are critical for mediating the disruptive effects SPS has on contextual extinction retention [71]. Given that enhanced neural activity in the BLA has been implicated in persistent fear expression [72, 73], enhanced BLA neural activity during extinction testing (stemming from changes in CB1 receptor signaling) could contribute to SPS-induced extinction retention deficits.

1.4.2 Limitations

In this study we used c-Jun to measure neural activity. This methodology allows for the measurement of neural activity in many brain regions at a common time point. This is advantageous, because changes in neural activity across many brain regions implicated in a behavioral phenomenon can be measured. However, animals have to be euthanized in order to assay c-Jun and c-Jun cannot be used to measure neural activity on small time scales. As a result, further research is needed to better understand how changes in dHipp, IL, and BLA neural activity, as well as changes in vmPFC $\longleftrightarrow$ BLA and vmPFC $\longleftrightarrow$ vHipp, lead to behavioral measures of extinction retention deficits in the SPS model.

1.5 CONCLUSION

The results of this study suggest that SPS disrupts extinction memory by disrupting vmPFC $\longleftrightarrow$ BLA and inhibited neural activity in the dHipp during extinction training. SPS may also disrupt retention of fear extinction in a manner that is not necessarily specific to fear extinction learning/memory. SPS exposure enhanced BLA neural activity, enhanced vmPFC $\longleftrightarrow$ vHipp and BLA $\longleftrightarrow$ dHipp functional connectivity, and disrupted inhibited neural activity in the dDG during extinction testing whether rats exposed to SPS formed an extinction memory or not.

Using c-Fos as a marker of neural activity, a previous study mapped circuits through which SPS may disrupt extinction retention [39]. There were notable differences between the results of Knox et al., [39] and the results of this study, which cannot be attributed to differences between groups of animals, because c-Fos and c-Jun assays were performed in the same groups of animals (i.e. c-Fos and c-Jun assayed in counter sections). For example, enhanced vmPFC $\longleftrightarrow$ BLA functional connectivity was observed when c-Jun was used to

map neural activity (see 1.3 Results), but not when c-Fos was used [39]. Enhanced BLA↔vHipp functional connectivity during extinction testing was observed when c-Fos was used to map neural activity [35], but in this study enhanced vmPFC↔vHipp was observed with c-Jun (see 1.3 Results). These results imply that there are sets of neurons across brain regions (i.e. circuits) that upregulate c-Jun and c-Fos with increased neural activity in a different manner. Such a hypothesis is consistent with the observation that all dimers of AP-1 are not expressed in all neuron types in the brain [74] and that subunits of AP-1 are not always regulated in an equivalent manner with cellular stimulation [40–42]. The significance of the differential regulation of c-Fos, c-Jun, and potentially other subunits of AP-1 during emotional learning and memory in stressed and non-stressed animals, requires further investigation.

Recent research has identified relatively novel neural substrates that are not traditionally considered being part of the fear extinction circuit, but are nonetheless critical for inhibiting fear memory [75–77]. Determining the effects of SPS in these relatively novel neural substrates is needed.

Finally, there were also a number of effects observed during extinction training in control rats that have been considered critical for extinction memory, but were observed in animals that did not undergo fear conditioning and thus never formed a fear or extinction memory. These includes enhanced neural activity in the vmPFC and disinhibited neural activity in the BLA. This suggests that some of the changes in neural activity believed to be critical for extinction memory are also observed with repeated stimulus presentation. Other studies have observed similar findings. Habituation to an unconditioned stimulus and repeated presentation of a CS prior to fear conditioning both enhance neural activity in the IL and increase retention of fear extinction [78, 79]. Future research examining how these non-fear extinction procedures result in changes in neural activity that inhibit expression of fear memory is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

BLA	Basolateral amygdala
CS	Conditioned stimulus
dHipp	Dorsal Hippocampus
Hipp	Hippocampus

IEG	Immediate early genes
IL	Infralimbic cortex
ITC	Intercalated cell masses of the amygdala
PBS	Phosphate buffered saline
PL	Prelimbic cortex
PTSD	Post traumatic stress disorder
SPS	Single prolonged stress
TBS	Tris buffered saline
UCS	Unconditioned stimulus
vHipp	Ventral Hippocampus
vmPFC	Ventromedial prefrontal cortex

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HIGHLIGHTS

- SPS exposure induces extinction retention (ER) deficits
- C-Jun was used to identify extinction relevant neural circuits sensitive to SPS
- SPS disrupted neural activity that was specific to fear extinction learning/memory
- SPS induced changes in neural activity that were not specific to fear extinction

Experimental Design

Figure 1.

Experimental design used in this study. All animals were euthanized 60 minutes after the start of behavioral sessions or after removal from the housing colony.

Figure 2.

Representative images in the vmPFC, dHipp, BLA, and vHipp obtained after performing c-Jun immunocytochemistry and scanning brain slides using the Li-cor Odyssey scanner.

vmPFC – ventromedial prefrontal cortex, IL – infralimbic cortex, PL – prelimbic cortex, dHipp – dorsal hippocampus, BLA – basolateral amygdala, vHipp – ventral hippocampus.

Figure 3.

Effects of stress and learning treatment on c-Jun levels in the fear extinction circuit. Blue bars represent control rats, while red bars represent SPS rats. Solid colors represent rats in the CS-fear (CS-F) treatment, while translucent bars represent rats in the CS-only (CS-O) condition. A) During extinction training and testing there was enhanced vmPFC c-Jun levels relative to baseline c-Jun levels. B) During extinction training, SPS enhanced c-Jun levels in the dCA3 and dDG regions of the dHipp in rats within the CS-F treatment. C) c-Jun levels were attenuated in the BLA during extinction training in all rats, but were enhanced in SPS rats during extinction testing. D) vHipp c-Jun levels were attenuated in all rats during

extinction training and testing. SPS had no effect on baseline c-Jun levels in any brain region. + indicates a significant one-sample t-test at the $p < .05$ level, while * indicates a significant stress effect at the $p < .05$ level.

Figure 4.

Representation of factor analyses performed in this study. In each box, the rotated component matrix, variance explained by each factor analysis, cartoon representation of the factor analysis, and schematic representation of the factor analysis is illustrated. Assignment of color (e.g. blue) and number (e.g. 1) to factor is arbitrary. Factor analyses revealed that functional connectivity in SPS rats was different to control rats under all treatments. Notably, there was vmPFC $\longleftrightarrow$ BLA connectivity in control rats during extinction training. Functional connectivity patterns during extinction testing was identical to that observed during baseline in control rats. These effects were absent in SPS rats. + means different from

respective baseline (e.g. control/CS-fear vs control/baseline), % means different from respective CS-only condition (e.g. control/CS-fear vs. control/CS-only), and * means different from respective SPS treatment (e.g. control/CS-fear vs. SPS/CS-fear).

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