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Single prolonged stress PTSD model triggers progressive severity of anxiety, altered gene expression in locus coeruleus and hypothalamus and effected sensitivity to NPY



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Abstract

PTSD is heterogeneous disorder that can be long lasting and often has delayed onset following exposure to a traumatic event. Therefore, it is important to take a staging approach to evaluate progression of biological mechanisms of the disease. Here, we begin to evaluate the temporal trajectory of changes following exposure to traumatic stressors in the SPS rat PTSD model. The percent of animals displaying severe anxiety on EPM increased from 17.5% at one week to 57.1% two weeks after SPS stressors, indicating delayed onset or progressive worsening of the symptoms. The LC displayed prolonged activation, and dysbalance of the CRH/NPY systems, with enhanced CRHR1 gene expression, coupled with reduced mRNAs for NPY and Y2R. In the mediobasal hypothalamus, increased CRH mRNA levels were sustained, but there was a flip in alterations of HPA regulatory molecules, GR and FKBP5 and Y5 receptor at two weeks compared to one week. Two weeks after SPS, intranasal NPY at 300 $\mu\text{g}/\text{rat}$, but not 150 μg which was effective after one week, reversed SPS triggered elevated anxiety. It also reversed SPS elicited depressive/despair symptoms and hyperarousal. Overall, the results reveal

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time-dependent progression in development of anxiety symptoms and molecular impairments in gene expression for CRH and NPY systems in LC and mediobasal hypothalamus by SPS. With longer time afterwards only a higher dose of NPY was effective in reversing behavioral impairments triggered by SPS, indicating that therapeutic approaches should be adjusted according to the degree of biological progression of the disorder.

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1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating long-lasting neuropsychiatric disorder which develops in a subset of individuals following exposure to a traumatic, often life-threatening, stress. The symptoms frequently display a delayed onset and may not be manifested in the immediate aftermath of the trauma. Some symptoms show partial remission and relapse while some are long lasting and often get progressively worse. The disorder can persist for more than 40 years. Therefore, it has become increasingly apparent that there is a vital need to evaluate the biological progression of PTSD and comorbid impairments by taking a staging approach to the biological mechanisms of the disease (McFarlane et al., 2017). It can reveal distinct therapeutic approaches to be utilized according to the degree of biological progression of the disease.

The hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis and the locus coeruleus/norepinephrine-autonomic (LC/NE) nervous system are key components of a highly conserved stress regulatory systems and are involved in the etiology and manifestation of PTSD (rev. in Hendrickson and Raskind, 2016; Yehuda, 2005). Corticotrophin releasing hormone (CRH) originating in the hypothalamic and extra hypothalamic brain regions plays a key role in orchestrating stress responses by acting as a neurohormone to initiate activation of the HPA axis and as a neuromodulator in the brain. HPA regulators such as glucocorticoid receptor (GR) and FK506 binding protein 5 (FKBP5) are associated with PTSD susceptibility. Alterations in the HPA axis are observed in PTSD, with the majority of patients displaying lower basal cortisol, enhanced glucocorticoid negative feedback and elevated CRH (Bremner et al., 1997; Baker et al., 1999; Yehuda, 2009).

The LC, the major noradrenergic (NE) nucleus in the brain, is rapidly activated by stress, releasing NE in many anatomically and functionally diverse brain regions, consolidating information and providing neuronal responses to stress by adjusting arousal, memory acquisition, attention, and vigilance to stressful environments (Aston-Jones et al., 1996; Sara, 2009; Valentino and Van Bockstaele, 2008). Patients with PTSD have exaggerated NE activity with its levels elevated in the cerebrospinal fluid, which is correlated with severity of the disease (Geraciotti et al., 2001). In addition, PET scans showed reduced norepinephrine transporter (NET) availability in the LC (Pietrzak et al., 2013). This was associated with anxious and arousal symptoms, notably with hypervigilance.

Animal PTSD models are very useful in evaluating the biochemical mechanisms mediating development of prolonged impairments, identifying biomarkers and testing putative therapeutic modalities. One of the best is the

single prolonged stress (SPS) rat model of PTSD (Liberzon et al., 1997; Pitman et al., 2012; Souza et al., 2017). Behavioral impairments triggered in rats by SPS correspond to many core symptoms of PTSD. SPS also triggers a variety of changes in stress-related neuroendocrine and molecular responses including negative HPA regulation, hypersensitivity of LC to novel stress, abnormal gene expression for CRH, GR, FKBP5 or CRHR1 in the hypothalamus, locus coeruleus, amygdala and hippocampus (Kohda et al., 2007; Laukova et al., 2014; Sabban et al., 2015).

Considerable evidence from human and animal studies implicate neuropeptide Y (NPY) in reducing or preventing harmful effects of stress. (reviewed in Sabban et al., 2016; Dumont and Quirion, 2014; Schmeltzer et al., 2016; Heilig, 2004; Kautz et al., 2017). Intranasal infusion of NPY was able to reach the brain, including the LC and hypothalamus (Serova et al., 2013; Sabban et al., 2016). It prevented or attenuated, by either prophylactic or early intervention, development of most of the SPS-triggered impairments (Serova et al., 2013; Sabban et al., 2016; Laukova et al., 2014). Moreover, when given one week after exposure to SPS stressors, intranasal NPY was able to reverse the already manifested anxiety and depressive-like behavior (Serova et al., 2014a). These findings supported the translational potential of intranasal NPY as a pharmacological intervention in PTSD patients.

However, most of these studies examined the PTSD-associated impairments after exposure to SPS stressors, as well as effectiveness of NPY as treatment, at one time point usually one week after the application of SPS stressors (reviewed in Souza et al., 2017). Few papers characterized the progression of PTSD-related symptoms at later times after the traumatic events (Serova et al., 2014a). Here, we begin to take a staging approach to examine the temporal trajectory of exposure to SPS stressors and to analyze whether sensitivity to NPY depends on the stage of the disorder. Since NPY is proposed to antagonize anxiogenic effects of CRH (Heilig, 2004), we focused on the NPY and CRH systems in both the hypothalamus and the LC.

2. Experimental procedures

2.1. Animals

All experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at NYMC and the USAMRMC Animal Care and Use Review Office. Male Sprague-Dawley rats (150-175 g) 6-7 weeks of age from Charles River (Wilmington, MA) were housed on 12 h light/dark cycle at $23 \pm 1^\circ\text{C}$ with ad libitum

access to food and water. After 14 days acclimation period, they were randomly assigned to the experimental or control groups.

2.2. Single prolonged stress (SPS)

The single prolonged stress (SPS) procedure was performed between 9 a.m. to 2 p.m. as previously described (Serova et al., 2013; Laukova et al., 2014; Sabban et al., 2014). Briefly, rats were immobilized for 2 h on a metal board by taping the limbs with surgical tape and restricting motion of the head, then subjected to forced swim for 20 min in a plexiglass cylinder (50 cm high, 24 cm diameter) filled to two-thirds with 24 °C water. They were dried and allowed to recuperate for 15 min before exposure to ether vapor until loss of consciousness. Following the SPS procedure, animals were left undisturbed (2 per cage) for 1 or 2 weeks.

2.3. Intranasal infusion

Rats, under light isoflurane anesthesia, were given intranasal infusion of NPY (custom synthesized by NeoScientific, Cambridge, MA) at 150 or 300 µg freshly dissolved in 20 µl distilled water or vehicle. They received 10 µl into each nostril, and the head kept tilted backwards an additional 15 s, as previously described (Serova et al., 2014a, 2013).

2.4. Behavioral evaluation

All behavioral measurements were analyzed by individuals blinded to the experimental groups. Anxiety behavior was tested on the elevated plus maze (EPM) apparatus (Stoelting, Wood Dale, IL) as previously described (Serova et al., 2013). The maze, 40 cm above the floor, had cross shaped platforms with two open arms with 2 cm high pleix-glass sides, and two closed arms with 40 cm high opaque walls. After 30 min acclimation to a room with dim light, rats were placed on the central platform facing an open arm and allowed to explore the maze for 5 min. Behaviors were recorded and analyzed using tracking software "Viewer 3.0" with a designated "Plug-in" program (Bioobserve). Entry into the open (OA) or closed (CA) arm was defined as entering with all four paws. Risk assessment was defined as the rat poking its head or trunk into an OA while its hind quarters were located in one of the CA. Anxiety index was calculated as: $1 - [(time\ spent\ in\ OA / total\ time\ on\ the\ maze) / 2 + (number\ of\ entries\ to\ the\ OA / total\ number\ of\ entries\ into\ OA\ and\ CA) / 2]$.

Depressive/despair behavior on the modified Porsolt forced swim test (FST) was carried out as previously described (Serova et al., 2013). Rats were put into a plexiglass cylinder (50 cm high, 24 cm diameter) filled two-thirds with 24 °C fresh water for 5 min and behavior was videotaped. The water was changed between animals. The time spent immobile was defined as the animal showing no movement, or only movements needed to keep its head above the water.

Hyperarousal was assessed by Acoustic Startle Response (ASR). ASR was measured in a sound-proof chamber (SR-LAB) (San Diego Instruments, San Diego, CA, USA). Animals were placed into a cylindrical enclosure designed for rats containing a platform connected to a piezoelectric accelerometer. The steadiness of the piezoelectric accelerometer was calibrated using a stabilimeter for consistent sensitivity among the chambers and over time. Sound levels within the test chambers were measured with a detachable probe sound level meter to ensure consistent presentation. After a 5-min accommodation period with white noise of 68 dB, animals were exposed to 10 repeats of 110 and 115 dB trials for 40 ms (total 20 trials) in random order with inter-trial intervals from 30 to 38 s. Background noise was set at 68 dB for the entire experimental protocol. Voltage data were collected and transferred to a computer using an automated software package (San Diego Instruments). Enclosures were carefully cleaned with soap and water in between animal measurements.

2.5. Determination of changes in mRNA levels for candidate genes in the mediobasal hypothalamus or the locus coeruleus

The rats used to determine molecular changes were not subjected to the behavioral tests. At the specified time following exposure to SPS stressors, rats were euthanized and the mediobasal hypothalamus, without arcuate nucleus, and the LC (9.2 –10.4 mm anterior to bregma) were isolated as previously described (Serova et al., 2014b). Total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, ML) and concentrations were determined using NanoDrop 2000 (Thermo Fisher Scientific, Pittsburgh, PA). Reverse transcription of 1000 ng of RNA for mediobasal hypothalamus and 600 ng for LC was performed with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) using an oligo dT primer. The cDNA (2 µl) was mixed with 12.5 µl of FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Indianapolis, IN) and 1 µl of one of the following primer sets from Qiagen: (Crh, PPR44803B); GR (NR3c2, PRR52805B); (Fkbp5, PPR51629B, Qiagen); (Crhr1, PPR44886F); (Npy, PPR44428A, Qiagen); (Npy1, PPR56359A, Qiagen), (Npy2r, PPR06816A); (Npy5r, PPR449006A, Qiagen); and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; forward 5'-TGGACCACCCAGCCAGCAAG-3', reverse 5'-GGCCCTCCTGTTGTTATGGGGT-3'), to a final volume 25 µl, and analyzed on a Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA) with QuantStudio™ Design & Analysis Software v. 1.4.1. Data were normalized to GAPDH mRNA (not altered by experimental conditions) and expressed as the relative fold changes calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

2.6. Immunohistochemistry

Immunohistochemistry of LC from rats perfused with 4% paraformaldehyde was performed with rabbit anti c-Fos (1:3000; Calbiochem, catalog number PC38) as previously described (Reyes et al., 2015). For quantification of c-fos

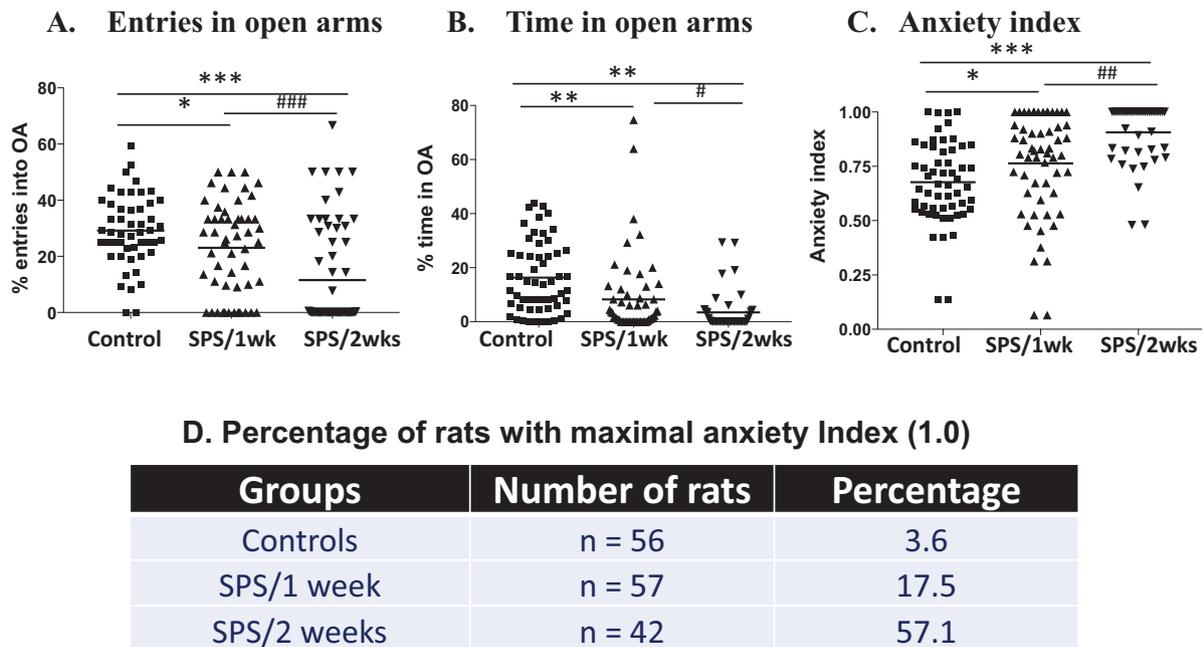


Fig. 1 Anxiety at one and two weeks after SPS stressors. A. Percentage of entries into the open arms (OA) of the EPM. B. Percentage of time in the OA of the EPM. C. Anxiety index; each data point represents an individual rat with mean (horizontal line). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Controls; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for SPS/1 wk vs SPS/2wks. D. Percentage of animals with maximal anxiety index (1.0) pooled from three separate experiments.

expression, every fourth coronal (120 μm apart) section was taken through the anteroposterior extent of the LC. The number of c-fos-labeled cells were counted unilaterally in six LC sections. A scorer blind to the experimental conditions identified and counted the number of c-fos-labeled cells. The average number of c-fos-labeled cells per animal was analyzed using Student's *t*-test (GraphPad Prism 6) to determine the significance between the two groups. For the control group, six rats were used while for the SPS group, seven rats were used.

2.7. Statistical analysis

Data were analyzed using Prizm 4 (GraphPad) and JMP10 (SAS Institute) software. All RNA data were normalized to mean levels of the controls for the individual experiment, which was taken as one. Data were analyzed by one-way ANOVA followed by Tukey Comparison of the means or Student's *t*-test for two groups. $P \leq 0.05$ was considered significant.

3. Results

3.1. Prolonged duration after traumatic stress

(a) Increases prevalence of extreme anxiety:

The manifestation of anxiety-like behavior on the EPM one and two weeks after exposure to SPS stressors was compared (Fig. 1). Rats were either unstressed or exposed to SPS stressors. They were undisturbed for one week

and tested on the EPM or left undisturbed for two weeks after SPS stressors and then tested on the EPM. Combining data from several separate cohorts (Fig. 1A-C) revealed a significant impact of interval following SPS on percentage of entries into OA ($F = 18$; $p < 0.05$), duration in OA ($F = 14$; $p < 0.001$) and anxiety index ($F = 6$; $p < 0.001$). Comparison of the means revealed that all SPS groups had fewer entries and lower duration in OA and higher anxiety index than the controls ($p < 0.001$ respectively, Fig. 1A and B). However, the SPS groups one or two weeks after SPS stressors differed in OA entries and duration ($p < 0.001$; $p < 0.05$) and anxiety index ($p < 0.01$). The percentage of rats with maximal anxiety index (no entries into the OA) increased from 3.6% in controls, to 17.5% one week and 57.1% two weeks after the SPS stressors (Fig. 1D).

(b) Prolonged induction of Fos.

To begin to understand the mechanism underlying these differences, in a separate cohort, rats were euthanized 2 weeks after SPS stressors, and processed for immunocytochemistry. Baseline Fos-like immunoreactivity in the LC was markedly elevated ($p < 0.05$) compared to unstressed controls processed at the same time (Fig. 2A and B).

(c) Changes in gene expression of CRH and NPY receptors in LC

Because the LC is a key brainstem region that is highly responsive to stressful stimuli that activate the HPA axis, and is involved in hyper-arousal, we assessed the changes in gene expression of the CRH and NPY systems in the LC two weeks after exposure to SPS stressors in two separate experiments by *t*-tests. CRHR1 mRNA was significantly above control levels in rats subjected to SPS ($p < 0.001$) (Fig. 2C).

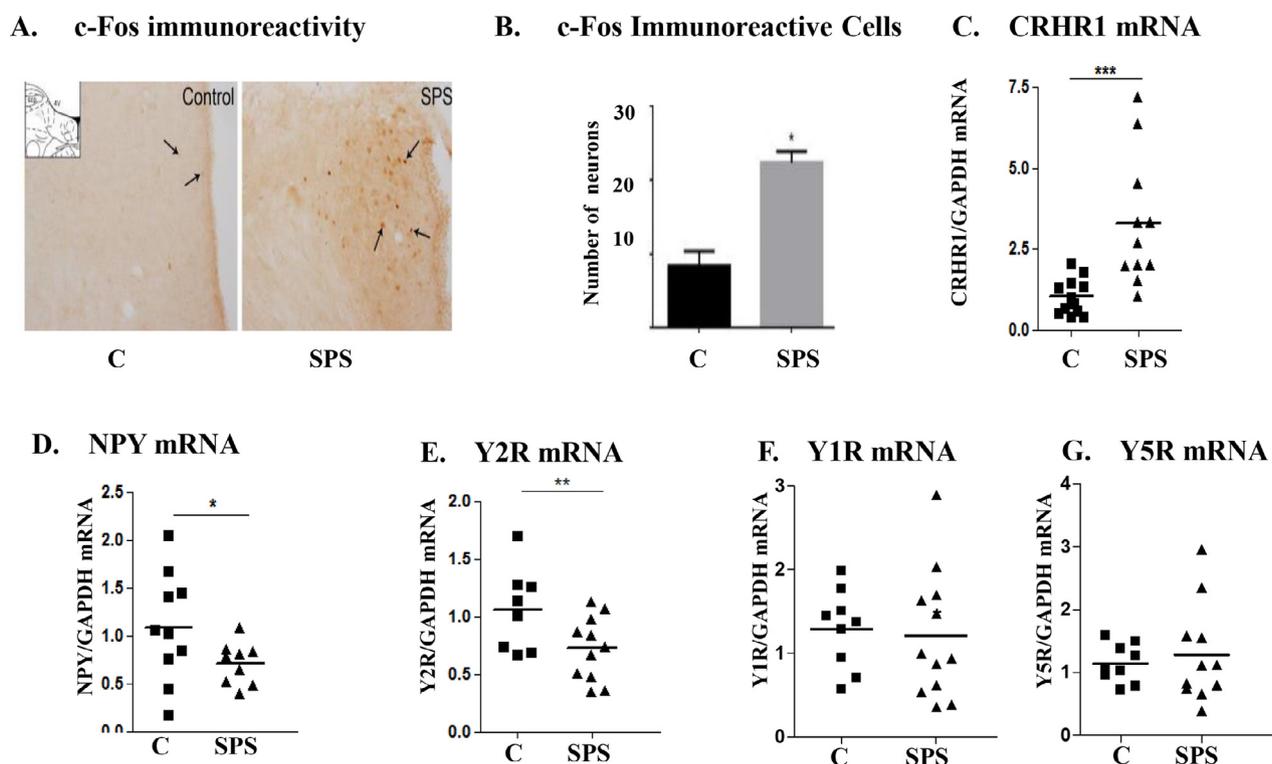


Fig. 2 Changes in gene expression in locus coeruleus at two weeks after exposure to SPS stressors. A. Fos immunoreactivity B. Number of Fos immunoreactive cells from six rats in the control group, and seven rats in the SPS group. C. CRH receptor 1 (CRHR1) mRNA levels. D. NPY mRNA levels. E. Y2R mRNA levels. Each data point represents an individual rat with mean (horizontal line). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by t -test of rats two weeks after exposure to SPS stressors compared to unstressed controls (C) of the same cohort of animals.

Next, we examined gene expression of NPY and its receptors. NPY mRNA levels were 20% lower than in the control group at this time point ($p < 0.05$, Fig. 2D). Y2R mRNA levels were reduced 25% after 2 weeks ($p < 0.01$, Fig. 2E). In contrast, there were no changes in levels of Y1R and Y5R mRNAs two weeks after SPS stressors (Fig. 2F and G).

(d) Changes in gene expression for CRH, GR, FKBP5 and NPY receptors in basomedial hypothalamus.

Two weeks after SPS stressors, CRH mRNA levels were increased over unstressed controls (Fig. 3A, $p < 0.001$). In contrast, the mRNA levels for GR (Fig. 3B) and FKBP5 (Fig. 3C) were decreased ($p < 0.01$). Since the effect of SPS on levels of gene expression of NPY receptors in the mediobasal hypothalamus were not previously examined, we analyzed their changes one and two weeks after SPS. Y1R mRNA levels were unchanged (Fig. 3D), while robust changes in Y2R and Y5R mRNAs were observed one week after the traumatic stress of SPS. After two weeks, only Y5R differed from the unstressed levels and was reduced to barely half the levels that were observed without SPS ($p < 0.01$) (Fig. 3E and F).

3.2. Reversal of anxiety, depressive-like behavior and hyperarousal two weeks after SPS stressors by intranasal NPY

(a) Anxiety

The ability of intranasal NPY to reverse SPS-triggered impairments in anxiety and depressive-like symptoms manifested two weeks after SPS stressors was tested. Rats exposed to SPS were treated with 150 μg /rat of NPY since this dose was previously shown to be effective in reducing SPS-elicited anxiety one week after SPS stressors (Serova et al., 2014a). To our surprise, intranasal administration of 150 μg /rat of NPY did not reverse anxiety symptoms manifested two weeks after SPS stressors. The percentage of entries into the OA on EPM was similar in rats given vehicle or NPY and lower than in the unstressed controls ($F = 7.2$ $p < 0.003$; Fig. 4A).

Since more animals displayed severe features of anxiety on the EPM two weeks after the SPS (Fig. 1) and reduced expression of NPY or selective NPY receptor mRNAs (Figs. 2 and 3), the dose of intranasal NPY was doubled in another cohort (Fig. 4). When tested on the EPM two days later, one way ANOVA revealed significant effect of treatment on entries into OA ($F = 20.5$, $p < 0.0001$, Fig. 4B); time spent in OA ($F = 6.7$ $p < 0.01$, Fig. 4C) and anxiety index ($F = 19$, $p < 0.001$, Fig. 4D). The NPY-treated rats were similar to unstressed controls in percentage of entries and time in OA as well as anxiety index. NPY treatment also increased frequency ($F = 8.4$, $p < 0.001$, Fig. 4E) and duration ($F = 6.7$, $p < 0.05$, Fig. 4F) of risk assessment to levels similar to controls ($p < 0.05$, Fig. 4E and F). There was a significant difference in locomotor activity as measured by track length on the EPM ($F = 3.2$, $p < 0.05$, Fig. 4G). Unstressed controls traveled longer distance than the SPS vehicle group. Admin-

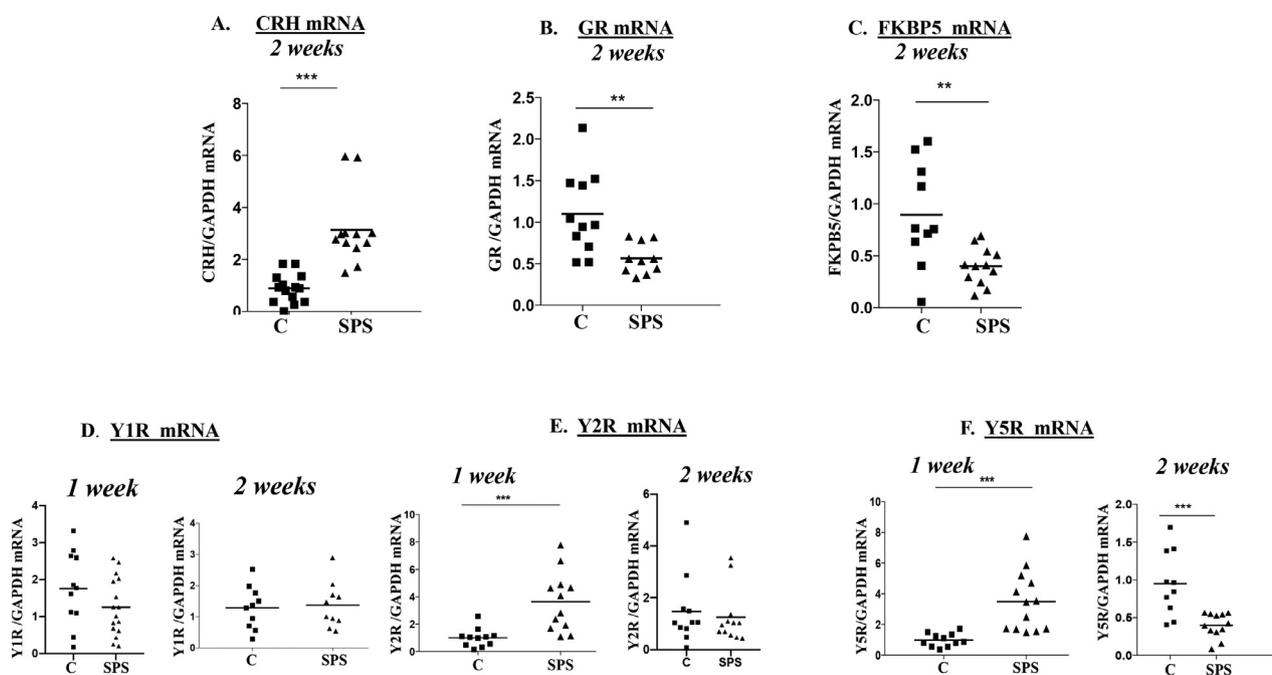


Fig. 3 Changes in gene expression in mediobasal hypothalamus after exposure to SPS stressors. Relative mRNA levels with control (C) taken as 1.0. for: A. CRH, B. GR, and C. FKBP5 two weeks after SPS stressors. D. Y1R. E. Y2R. F. Y5R mRNA levels. at one and two weeks after the SPS. Each data point represents an individual rat with mean (horizontal line). ** $p < 0.01$, *** $p < 0.001$ of rats exposed to SPS stressors compared to control (C) group at the same time point.

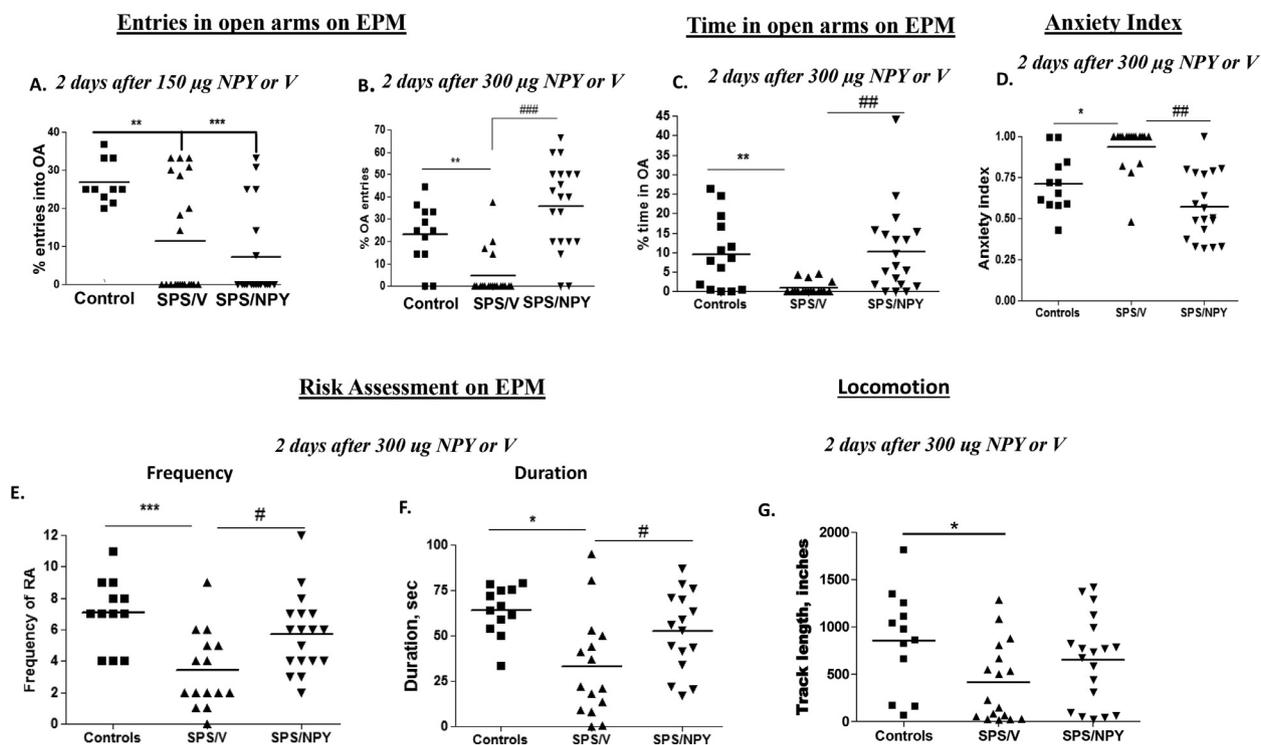


Fig. 4 Effect of intranasal administration of NPY to reverse symptoms of anxiety. Results on the EPM shown for entries into open arms in rats administered: A. 150 µg/rat of NPY (SPS/NPY) or vehicle (SPS/V) two weeks after SPS stressors compared to unstressed controls (Controls). B-G Rats were administered 300 µg/rat of intranasal NPY or vehicle 2 weeks after SPS stressors and tested on EPM for: B. entries into open arms; C. time in open arms; D. anxiety index E. Frequency; F. duration of risk assessment on the EPM and G. locomotion. Each data point represents an individual rat with mean (horizontal line). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for SPS/V vs SPS/NPY.

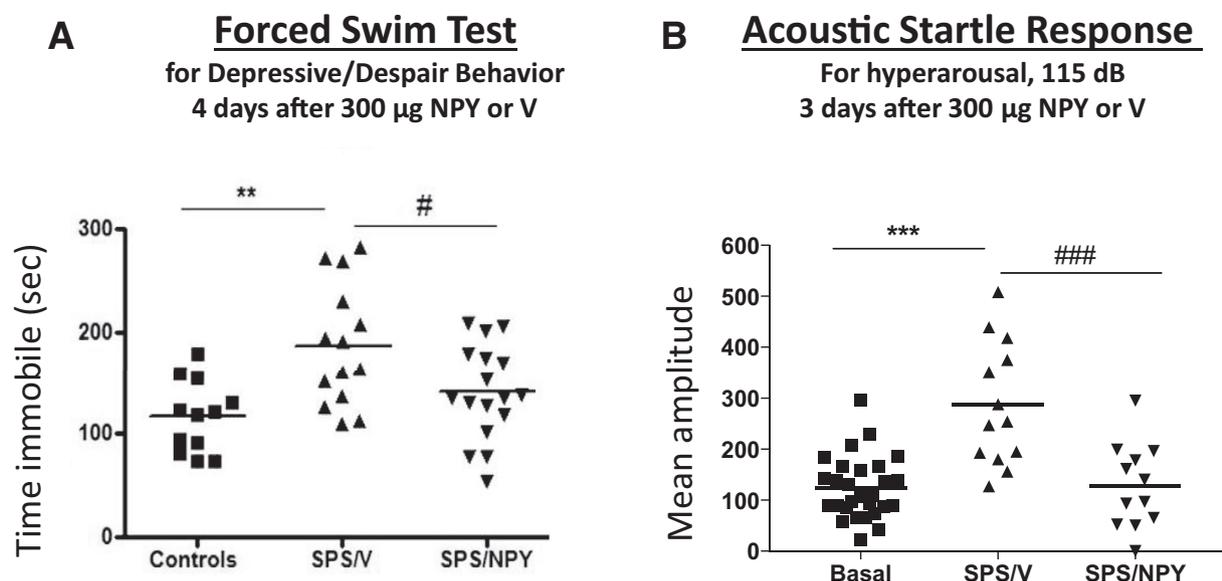


Fig. 5 Effect of intranasal administration of NPY to reverse symptoms of depression and hyperarousal. A. Two weeks after SPS stressors, rats were administered 300 µg/rat of intranasal NPY (SPS/NPY) or vehicle (SPS/V) and after two days tested on EPM. Two days later they were subjected to the forced swim test (FST). B. Separate cohort of rats were administered intranasal NPY or vehicle two weeks after SPS stressors and 3 days later tested for Acoustic Startle Response. Each data point represents an individual rat with mean (horizontal line). ** $p < 0.01$, *** $p < 0.001$ compared to Control; # $p < 0.05$, ### $p < 0.001$ for SPS/V vs SPS/NPY.

istration of intranasal NPY did not have a significant effect on the locomotion.

(b) Immobility on FST

Immobility times on FST, were similarly elevated over unstressed controls ($F = 7.1$, $p < 0.01$). Tukey comparisons of the means revealed significant differences between the SPS/V group and controls ($p < 0.05$ and SPS/NPY ($p < 0.01$) (Fig. 5A). Thus a higher dose of NPY reduced immobility time in FST which did not differ significantly from unstressed controls, and was lower than in SPS treated animals given only vehicle (Fig. 5A).

(c) Acoustic startle response (ASR)

Administration of intranasal NPY was also able to reverse the hyperarousal as tested by ASR. Two weeks after exposure to SPS, rats were administered either intranasal 300 µg NPY/rat (SPS/NPY) or vehicle (SPS/V). Three days later, there were significant differences in ASR ($F = 18.6$, $p < 0.001$). The group given vehicle displayed elevated startle ($p < 0.001$), while those given intranasal NPY had ASR levels similar to the basal pre-SPS levels and lower than those only given vehicle ($p < 0.001$) (Fig. 5B).

4. Discussion

The results of this study demonstrate a time-dependent progression in the development of anxiety symptoms in the SPS model of PTSD that was accompanied by significant molecular impairments in gene expression in CRH and NPY systems in the LC and mediobasal hypothalamus. Severe anxiety, as indicated by limited duration and entries into open arms of

EPM and high anxiety index, was much more pronounced at two weeks, compared to one week following the traumatic stress, suggesting a delayed worsening of symptomatology. At the two week time point, the beneficial effect of intranasal NPY in reversing PTSD-related behaviors required double of the dose that had been shown to be effective one week following SPS stressors. In addition to anxiety, intranasal NPY also reversed SPS elicited immobility on the FST and hyperarousal symptoms.

The results demonstrate the value in taking a staging approach to studying development of PTSD, and suggest that SPS might be an appropriate model for delayed-onset PTSD. Although one week after exposure to SPS stressors some of the animals displayed severe anxiety (no entry into open arms on EPM, anxiety index of one), the remainder did not differ from unstressed controls at this time point. However, two weeks after the traumatic stress, an additional 40% (57% in all) of the animals displayed severe anxiety, indicating delayed onset of these symptoms (Fig. 1, C and D).

Our findings demonstrate pronounced changes in gene expression in both the LC and mediobasal hypothalamus and several behavioral impairments 2 weeks following a single exposure to SPS stressors. In contrast, most studies have examined impairments one week after SPS stressors (reviewed in Souza et al., 2017). A study claimed that the SPS model is not suitable after 7 days as the impairments are no longer observed (Wu et al., 2016). These discrepancies may be due to experimental variations in the SPS protocol used, including strain of rats, severity of the first stressor, water temperature for forced swim, recuperation period, acclimation period, and number of rats per cage. In this regard, with a somewhat modified SPS protocol, Toledano and Gisquet-Verrier (2014) observed that behavioral impairments were

FKBP5 gene expression between one and two weeks post-SPS stressors. Although mRNAs for GR and FKBP5 were increased over levels in unstressed rats in the early stage (seven days after the SPS; [Laukova et al., 2014](#)), they were down regulated by 2 weeks. Ligand activated GR plays a crucial role in direct glucocorticoid feedback by repressing CRH biosynthesis and thus enabling appropriate termination of the stress response. GR sensitivity to corticosterone, at least partially, depends on FKBP5. Functional variations in the FKBP5 gene are associated with biologically distinct subtypes of PTSD and predicting the severity of its onset ([Mehta et al., 2011](#)). When FKBP5 is bound to the GR via Hsp90, the receptor has lower affinity for its ligand and remains in the cytoplasm instead of translocation to the nucleus. At 2 weeks after SPS, the persistent activation of CRH transcription may be due to decreased levels of FKBP5 and, as a consequence, more translocation into the nucleus. Conversely, the reduced levels of FKBP5 mRNA may result from the reduction in GR gene expression, since the transcription of the *Fkbp5* gene is regulated by GR in an ultra-short loop.

The PVN receives NPY mainly from the hypothalamic arcuate nucleus which innervates numerous PVN-CRH neurons ([Danger et al., 1990](#)) acting most probably via Y1R, Y2R and Y5R, Gi/Go coupled receptors ([Wolak et al., 2003](#)). NPY has a bimodal concentration-dependent effect on neuronal discharge of PVN neurons with excitatory responses at low concentrations, which may be mediated by Y1R and inhibitory responses at high concentrations mediated most probably by Y5R ([Aramakis et al., 1996](#)). In unstressed rats, intracerebroventricular infusion of NPY into the brain increased CRH mRNA levels, predominantly via Y1R ([Dimitrov et al., 2007](#)). However, in stressed animals NPY promotes adequate termination of stress therefore reducing exposure of the body to high levels of stress hormones and subsequently protects against harmful stress-related impairments ([Serova et al., 2013](#); [Palkovits, 2008](#); [Heilig, 2004](#)).

Here we found that SPS triggered time-dependent changes in gene expression for several NPY receptors subtypes in the mediobasal hypothalamus. Despite its effects on anxiety, we did not detect changes in Y1R mRNA expression in the mediobasal hypothalamus at any of the time points. However, Y5R mRNA was significantly altered after one and two weeks. Since the functions of Y1R and Y5R on anxiety behavior are similar ([Sørensen et al., 2004](#)) and higher levels of both receptors are associated with reduced anxiety ([Dumont and Quirion, 2014](#)), we speculate that decreased Y5R at two weeks might contribute to severity of anxiety. However, the ratio Y1R/Y5R might also play an important role as conditional deletion Y1R from Y5R expressing neurons caused an increase in anxiety behavior ([Longo et al., 2015](#)). Interestingly, like GR and FKBP5, there was also a flip in the gene expression of Y5R which was up-regulated one week after SPS and down regulated after two weeks ([Fig. 5](#)). The Y2 receptor subtype was markedly increased, but only at one week following the SPS. Potential Y2R signaling may be anxiogenic since, as an autoreceptor, its activation leads to a reduction in NPY release via a negative feedback loop ([Caberlotto et al., 2000](#)).

Further studies need to be performed to determine how the changes observed in gene expression are reflected in protein expression and their specific localization. Overall, the results clearly demonstrate SPS elicits a time-dependent

progression in the development of anxiety symptoms and molecular changes in gene expression for CRH and NPY systems in the LC and mediobasal hypothalamus.

The results indicate a severe dysbalance between CRH and NPY systems with the CRH system prevailing at the time when the rats display the extreme anxiety, which is overcome with a higher dose of NPY. At this time point intranasal administration of 150 µg NPY, which could reduce, and even reverse, several SPS-triggered behavioral impairments one week after SPS stressors, was not effective. NPY has been proposed to antagonize the response to CRH ([Heilig, 2004](#)), possibly by reducing the CRH elicited increase in cAMP. At the longer time (2 weeks) after the traumatic stress of SPS, the increased gene expression of the CRHR1 in the LC as well as enhanced CRH from the hypothalamus may not be as easily overcome by NPY. At this time point a double dose of NPY was able to reverse this severe anxiety. While these results indicate that higher dose of NPY is required at longer times after SPS, this needs to be confirmed with several doses administered to the same cohort of animals.

NPY, at this dose, also reduced the SPS-elicited rise in immobility on the FST. Enhanced immobility on the FST has been interpreted as representing depressive/despair behavior or more accurately, shift from active to passive coping behavior ([de Kloet and Molendijk, 2016](#)). In our studies the FST also represented an element of re-experiencing since forced swim in the same cylinders is one of the SPS stressors. Hyperarousal, as measured by acoustic startle, was also reduced by this dose of NPY. These results also emphasize that therapeutic treatment with NPY, or other medications to reverse or improve PTSD symptomology, should be applied according to the degree of biological progression of the disorder.

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Contribution of authors

Lidia I Serova played a key role in designing, carrying out the experiments, supervising, statistical analysis and writing the manuscript, Chiso Nwokafor was involved in the performance of SPS and reversal of ASR by NPY, and in editing the manuscript, Elisabeth J. Van Bockstaele and Beverly A. S. Reyes performed the immunocytochemical analysis for the LC, and participated in the discussion, Xiaoping Liu participated in SPS and behavioral analyses, Esther L Sabban was responsible for the planning, supervision of the experiments and analysis, interpretation and writing the manuscript. All authors contributed and have approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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