Comparative Effects of Intranasal Neuropeptide Y and HS014 in Preventing Anxiety and
Depressive-like Behavior Elicited by Single Prolonged Stress.

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Abstract

Stress triggered neuropsychiatric disorders are a serious societal problem. Prophylactic treatment or early intervention has great potential in increasing resilience to traumatic stress and reducing its harmful impact. Previously, we demonstrated proof of concept that intranasal administration of neuropeptide Y (NPY) or the melanocortin receptor four (MC4R) antagonist, HS014, prior to single prolonged stress (SPS) rodent post-traumatic stress disorder (PTSD) model, can prevent or attenuate many PTSD associated impairments. Here, we compare effects of NPY or HS014 given 30 min before or immediately after SPS stressors on development of anxiety, depressive-like behavior and associated biochemical abnormalities. SPS triggered anxiety on elevated plus maze (EPM) was reduced by intranasal administration of 100 µg NPY and to even greater extent HS014 (3.5 ng or 100 µg). The SPS-elicited depressive-like behavior on forced swim test was prevented with 100 µg NPY or the high dose HS014. Combined administration of low HS014 and NPY, ineffective by themselves, prevented development of depressive-like behavior. Reductions in stress triggered activation of locus coeruleus/noradrenergic system and HPA axis were observed with both HS014 and NPY. In contrast to NPY which has been showed earlier, infusion of HS014 immediately after SPS did not prevent the development of anxiogenic behavior on EPM. However, HS014 given after SPS stressors effectively even at very low dose, prevented development of depressive-like behavior. Thus, both MC4R antagonist and NPY, alone or combined, have potential for prophylactic treatment against traumatic stress triggered anxiety or depressive-like behaviors, while NPY has more widespread potential for early intervention.

Key Words: Single Prolonged Stress; NPY; HS014; Melanocortin Four Receptor; Depression; Anxiety; PTSD.

Abbreviations: ACTH, adrenocorticotropin hormone; CA, closed arms; CRH, corticotrophin-releasing hormone; EPM, elevated plus maze; GAPDH, glyceraldehyde-3-phosphate; HPA, hypothalamic-pituitary-adrenal; FST, forced swim test; GR, glucocorticoid receptor; LC, locus coeruleus; MC4R, melanocortin 4 receptors; NE, norepinephrine; OA, open arms; SPS, single prolonged stress; PTSD, Post-traumatic stress disorder; TH, tyrosine hydroxylase

1. Introduction

Stress elicits a cascade of neuronal, endocrine, and behavioral responses that promote homeostatic adaptations to changing or threatening environments. However when stress is unpredictable and extreme it can lead to pathological maladaptive responses within brain stress-integrative systems and development of stress-related psychiatric disorders including post-traumatic stress disorder (PTSD) and depression. Prophylactic treatment or early intervention has great potential for increasing resilience to traumatic stress and reducing its harmful impact (1, 2). Since PTSD is generally associated with a specific trigger stressor, it is especially suitable for early pharmacological intervention.

Animal models and limited human studies have revealed some potential treatments for early intervention, such as high dose glucocorticoids, neuropeptide Y, melanocortin 4 receptor antagonist, cannabinoid receptor agonists and CRH1 antagonist to prevent or ameliorate development of PTSD associated neuropathology (3-6). Several neuropeptides have been delineated regarding their potential significant clinical relevance in the treatment of stress-related mood disorders (7-9). Among them are neuropeptide Y (NPY) and HS014, an antagonist for the melanocortin four receptor (MC4R). Both can regulate stress-responsive systems - the hypothalamic pituitary adrenal (HPA), the CNS noradrenergic and the sympathetic nervous as well as associated behaviors [rev. in (10-14)]. Our previous results revealed that rats receiving NPY by intranasal infusion prior to or immediately after exposure to single prolonged stress (SPS) showed anxiety- and depressive-like behavior comparable to controls and significantly decreased compared to vehicle treated rats (15) confirming its significance as possible stress-reducing PTSD-preventive treatment. NPY infusion was also beneficial when given even a week later when the PTSD-like impairments are manifested (16).

Similarly, intranasal administration of the MC4R antagonist HS014 in high (100 µg) and low (3.5 ng) doses prior to exposure to SPS ameliorated many components of anxiety-like behavior while only the high dose had a beneficial effect on depressive-like behavior manifested by reduction of immobility time in FST (17).

In the present study we performed analysis of beneficial properties of intranasally infused NPY and HS014 as prophylactic or early intervention treatments against development of PTSD co-morbid behavior and biochemical abnormalities. Since several studies demonstrated

interaction between NPY and the melanocortin systems (18-20) we also examine whether there are synergistic effects between them.

2. Materials and methods

2.1. Chemicals

Neuropeptide Y was custom synthesized by NeoBioSci (Cambridge, MA, USA) to 98.9% purity (GLP purification for animals) and was stored lyophilized in 0.4mg aliquots until use. HS014 was purchased from Tocris Bioscience, R&D Systems, Inc (Minneapolis, MN, USA).

2.2. Animals

Male Sprague-Dawley rats from Charles River (Wilmington, MA, USA) weighing 150-160 g at arrival were housed in a barrier area on 12 hr light/dark cycle at $23 \pm 2^{\circ}\text{C}$ with *ad libitum* access to food and water. Animals were acclimated for 14 days, four per cage, and then randomly assigned to the experimental groups and housed two per cage. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at NYMC.

2.3. Intranasal administration

A 10 μl volume of freshly dissolved peptide or vehicle (distilled water) was infused into each nare with pipette and disposable plastic tip under light isoflurane anesthesia either 30 min prior to the SPS exposure or at the end of the SPS procedure when the rats were still under the influence of the ether. Extreme care was taken to avoid contact with the intranasal mucosa. Following the infusion, the head of the animal was held in a tilted back position for approximately 15 s to prevent loss of solution from the nares.

2.4. Single prolonged stress (SPS)

The SPS animal model of PTSD combines psychological, physiological and endocrine components of stress (21-23). The SPS was performed as previously described (15, 17). Briefly, rats were first immobilized for 2 hrs on a metal board by taping the limbs with surgical tape and restricting the motion of the head. Following immobilization, rats were immediately subjected to forced swim (FS) for 20 min in a plexiglass cylinder (50 cm height, 24 cm diameter, Stoelting, Wood Dale, IL, USA) filled to two-thirds with 24° C fresh water. The animals were dried and allowed to recuperate for 15 min and then exposed to ether vapor until loss of consciousness. The SPS procedure was performed between 9AM to 2PM. In some experiments, animals were sacrificed 30 min after application of the last SPS stressor. In other experiments, following SPS stressors animals were housed two per cage and left undisturbed for 7 days to allow for PTSD-like symptoms to manifest and then were sacrificed or subjected to behavioral testings. Tissues were collected 30 min or 7 days following the application of SPS stressors.

2.5. Behavioral tests

All behavior tests were performed in specifically designated rooms in the animal facility seven days after SPS stressors. Unstressed control rats were handled for 5 min for three consecutive days prior to the EPM test. The animal's behavior was video captured and analyzed using tracking software (Bioserve, Viewer 3.0). Behavior scoring was performed by a trained individual blinded to the experimental groups. Groups were staggered during the testing, and when testing was carried out on more than one day, all groups were equally representing on each test day.

2.5.1. Elevated plus maze (EPM)

Anxiety-like behavior was tested on EPM as previously described (15). After a 30 min acclimation period rats were allowed to explore the maze for 5 min. Several parameters such as duration and entries in open (OA) and closed arms (CA); time and frequency of risk assessment (RA); track length were analyzed using tracking software "Viewer 3.0" with a designated "Plug-in" program (Biobserve). Arm entry was defined as entering an arm with all four paws. Risk assessment was defined as the rat poking its head or trunk into an open arm while its hind

quarters were located in one of the closed arms. Anxiety index was calculated as previously described (15).

2.5.2. Forced swim test (FST)

The FST was performed in a plexiglass cylinder with water at 24⁰C, as for SPS stressors, for 5 min. Immobility was scored when the animal showed floating in the water without struggling, and making only those movements necessary to keep the head above the water.

2.6. Biochemical measurements

2.6.1. Plasma corticosterone and ACTH levels

Plasma ACTH levels were determined as previously described using ACTH ELISA kit (MyBioSource, San Diego, CA, USA) or corticosterone double antibody ¹²⁵I RIA Kit (MP Biomedical Diagnostics, Solon, OH, USA) according manufactures' protocols (24).

2.6.2. Tyrosine hydroxylase (TH) mRNA levels

Reverse transcription of total RNA isolated from the LC was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol, using an oligo dT primer as in our previous experiments (24). The quantitative Real-Time PCR was carried out using FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Indianapolis, IN, USA) and rat TH (forward 5'-CCGGTCTACTGTCCGCCCGT-3', reverse 5'-TCATGGCAGCAGTCCGGCTC-3') and GAPDH (forward 5'-TGGACCACCCAGCCCAGCAAG-3', reverse 5'-GGCCCCCTCCTGTTGTTATGGGGT-3') primers.

2.6.3. Western blot analysis for glucocorticoid receptors (GR)

Western blots were performed as previously described (24). Briefly, total protein from hippocampus was separated on 4-20% Tris-HCl gradient precast CriterionTM gel (Bio-Rad,

Hercules, CA, USA) and membranes were incubated with primary anti-glucocorticoid receptor antibody (GR; M-20; sc-1004, Santa Cruz, Santa Cruz, CA, USA; 1:500) and then with secondary anti-rabbit antibody (sc-2004; Santa Cruz, Santa Cruz, CA, USA; 1:3,000). Films were scanned with Bio-Rad G-800 Calibrated Densitometer within the linear range and optical density of individual bands was evaluated by PCBAS 2.08e software (Raytest, Stanbenhardt, Germany).

2.7. Statistical analysis

Data were analyzed using JMP 9 (SAS Institute) and Prism 4 (GraphPad) software. The results were analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test for differences among the groups. Two way ANOVA for main effects of treatment (V, HS014 or NPY) and time (different intervals) was used for analysis of immobility time during forced swim as part of the SPS stressors.

3. Results

3.1. Infusion of NPY and HS014 prior to SPS stressors

3.1.1. Effect on SPS-triggered anxiety

Rats were given intranasal infusion of either NPY (100 µg), HS014 (3.5 ng or 100 µg) or vehicle and 30 min later subjected to SPS stressors. After seven days undisturbed they were tested on the EPM along with a parallel group of rats which were not stressed (15, 17). Although anxiety, tested on EPM, was significantly reduced in animals given HS014 or NPY prior to SPS, the results were not exactly the same (Table 1). Compared to vehicle treated animals, a single infusion of either NPY or HS014 prior to SPS led to more entries into the open arms (OA), less entries into the closed arms (CA) and reduced anxiety index and more risk assessment behaviors. However, while NPY did not significantly alter the duration in the OA or CA, animals given HS014 spent more time in the OA and less time in the CA compared to the vehicle treated group. Grooming behavior in the EPM was not altered by NPY, while it was reduced in animals given the high dose of HS014.

3.1.2. Effect on despair or depressive-like behavior

The immobility during forced swim was measured at two times of the SPS procedure: (1) acute effect during the traumatic stress; (2) delayed effect 7 days later. The immobility at 5 min intervals during the 20 min forced swim (FS) part of the SPS stressors is shown in Fig. 1 in animals pretreated 30 min before SPS stressors (2 hr 30 min before the FS) with several concentrations of HS014, NPY or the vehicle. Two way ANOVA revealed significant influence of main factors: interval ($F_{3,201} = 3.5$, $p < 0.05$) and treatment ($F_{4,201} = 21$, $p < 0.0001$). Tukey's post-test showed that in the first 5 min interval there are significant differences between 100 µg HS014, NPY and vehicle treated groups ($p < 0.01$ for both treatments). The effect of the high dose HS014 was more long lasting as the differences in the floating time remained significantly different from vehicle during the 2nd ($p < 0.01$), 3rd ($p < 0.01$) and 4th ($p < 0.05$) interval only in the group given 100 HS014. In contrast, infusion of 3.5 ng HS014 or 50 µg NPY did not influence immobility time which was comparable to the group given vehicle.

A delayed effect was determined on the forced swim test (FST) performed after a seven day undisturbed period following exposure to the SPS stressors (Fig. 2). Here also administration of 3.5 ng of HS014 was unable to reduce the SPS-elicited increase in immobility. However,

animals given 100 µg HS014 or NPY had immobility times similar to control animals, although 100 µg HS014 treated animals were even better than those given NPY showing significantly lower immobility time ($p < 0.01$).

Since HS014, a MC4R antagonist, and NPY are likely to utilize different mechanisms to alter the SPS triggered behavior, we examined if combined treatment with low doses which are ineffective by themselves might reduce the SPS triggered depressive-like or despair behavior measured on the FST (Fig. 2B). Animals given intranasal infusion of either 50 µg NPY or 3.5 ng HS014 were immobile for longer on the FST than unstressed controls ($p < 0.01$ for both treatments). However combination of the peptides was very effective to reduce immobility time compared to each of them given separately ($p < 0.05$ or 0.01) and did not differ significantly from the unstressed controls.

3.1.3. Effect on noradrenergic system and HPA axis

PTSD is associated with dysregulation of the central and peripheral noradrenergic system and the HPA axis (25-27). Therefore, we examined changes in these systems in animals given HS014 or NPY prior to exposure to SPS (15). Acute effects were examined 30 min after SPS stressors (Fig. 3). SPS triggers a rise of mRNA levels for the major rate limiting enzymes in norepinephrine biosynthesis tyrosine hydroxylase (TH) in the locus coeruleus. There were decreased TH mRNA levels in animals receiving intranasal NPY or HS014 ($p < 0.05$) compared to rats treated with vehicle (Fig. 3A). Interestingly, the low dose of 3.5 ng HS014 was especially effective. As shown on Fig. 3B and Fig. 3C, at this time point compared to vehicle there was also relatively small, but significant, decrease in plasma corticosterone ($p < 0.05$) and ACTH ($p < 0.05$) levels in animals given NPY. With HS014, similar changes were only observed in corticosterone, not ACTH, and only with the high dose of HS014.

Long term changes were examined 7 days after SPS stressors, at the time when anxiety- and depressive-like behaviors were evaluated. SPS triggered induction of GR levels in the hippocampus of vehicle treated animals. However GR levels in the hippocampus did not differ from control in animals administered either low (3.5 ng) or high (100 µg) dose of HS014 or 100 µg NPY prior to SPS (Fig. 4).

3.2. Treatment post-SPS stressors: Effects on anxiety- and depressive-like behavior

We have previously shown that intranasal infusion of 150 µg (only concentration tested) NPY immediately after exposure to SPS stressors, or even seven days afterwards when the SPS elicited impairments are manifested, can improve performance on the EPM and FST (15, 16).

Here, we examined whether infusion of HS014 after the SPS stressors will also lead to similar improvements. For these experiments, rats were infused with either vehicle or HS014 (3.5 ng or 100 µg) while still under the influence of ether (the last SPS stressor). After a week undisturbed, animals were tested on the EPM (Fig. 5) and FST (Fig. 6) and compared to unstressed controls.

In sharp contrast to the reduced anxiety when given 30 min prior to SPS stressors, HS014 treated animals did not differ from the vehicle treated in percentage of entries or duration in the open or closed arms. All groups subjected to SPS, whether with vehicle or either dose of HS014 significantly differed than the unstressed controls. The SPS triggered reduction in duration and frequency of risk assessment was prevented with the high, but not low dose, of HS014. However, as we previously observed, rats treated with NPY in the similar experimental paradigm did not differ in these behavioral features from untreated controls (15). The reaction of sympathetic nerve system in animals with both doses of HS014 on novel situation of EPM, manifested in number of droppings, was close to normal observed in unstressed control and significantly lower than in animals with vehicle ($p < 0.01$).

Here, in contrast, the animals given HS014 immediately post-SPS stressor, like those given NPY, displayed improvements in depressive-like behavior. Their immobility time was reduced compared to vehicle infused animals and was comparable to that of unstressed control. This improvement was observed with both doses of HS014 (Fig. 6).

4. Discussion

The results reveal that MC4R antagonist, HS014, and NPY have potential for prophylactic treatment to prevent development of anxiogenic or depressive-like behaviors, when given prior to exposure to traumatic stress. Administration prior to SPS of a wide range of HS014 concentrations appeared to be somewhat more effective than NPY in preventing development of anxiogenic behavior. However, the modulation of depressive-like behavior required a high dose of HS014. Combined administration of HS014 and NPY at low doses, ineffective by themselves, prevented development of depressive-like behavior. Reductions in stress triggered activation of central noradrenergic system and HPA axis were observed with both HS014 and NPY. In contrast to results with prior to SPS infusion of HS014 early intervention with HS014 immediately after traumatic stress did not prevent the development of anxiolytic behavior on EPM. However, HS014 given after SPS stressors was effective, even at very low dose, to prevent development of depressive-like behavior.

Comparisons of beneficial effects of NPY and HS014 revealed common features and important differences in their properties as treatments against development of harmful consequences of traumatic stress which depend on the dose and time of intranasal administration. For anxiety-like behavior the key factor was time of intranasal infusion. Both NPY and HS014 given prior to application of SPS stressors, with slight variation in a favor of HS014, successfully prevented anxiety-like behavior. However their effectiveness was radically different when they were infused post SPS stressors. Although single intranasal NPY infusion in this time point successfully block development of many features characterizing anxiety behavior (15) both doses of HS014 failed to produce similar effect. The results suggest that activation of the MC4R is involved in the effect of traumatic stress on anxiety during the “initiation” phase, and thus need to be antagonized prior to or near the beginning of the traumatic stress. In this regard, another potential prophylactic intervention, a CRH antagonist, was also more effective when given prior, rather than immediately after traumatic stress in the predator stress PTSD model (6).

Overall animals given HS014 post-SPS stressors were similar to the vehicle treated animals on the EPM. The only exemption was rats given 100 µg HS014 whose risk assessment was comparable to the unstressed controls. Administration of HS04 post-SPS stressors led to less defecation on the EPM, which suggests that the sympathetic nerve system in rats given HS014 in

this time point is more adaptive to minor stress than in rats given vehicle instead. However it requires further investigation.

The preventive properties of these peptides on SPS triggered depressive-like behavior were also influenced by time of administration. Given post-SPS, NPY and both doses of HS014, reduced to the same extent "giving up" behavior in FST. However the consequences were different with infusion prior to SPS. First, although both drugs in relatively high doses prevented long term SPS-elicited increased floating time, the degree of reduction of immobility time was better with high dose of HS014. Secondly, neither low doses of NPY nor HS014 were effective. The anti-depressive effect of peptides was evident during FS stress as a part of SPS. The acute antidepressant effect of drugs usually are analyzed for 5 min of FST and considered as an index of coping strategy which is essential for adaptation to stressful conditions (28-30). Thus, the first 5 min interval of forced swim stress following immediately after two hours of immobilization can be related to degree of depression induced by only this stressor. The results clearly demonstrate for the first time that only rats with NPY and HS014 in higher concentrations struggled less unlike animals with lower doses or vehicle, suggesting that intranasal infusion of 100 µg either NPY or HS014 prior to immobilization helped animals to overcome immobilization stress. Another very important finding is that only 100 µg of HS014 given prior to immobilization has long lasting effect during the entire 20 min of forced swim stress, perhaps since a substantial level will remain even if some is degraded during the procedure.

The results with infusion of HS014 after the SPS stressors show dissociation from the effect on anxiety and depressive-like behavior. While these behaviors are often comorbid, there are other instances in which they are independent. It remains to be determined whether delivery of HS014 can also modulate the development of other PTSD associated behaviors, such as hyperarousal, fear memories, substance abuse etc.

Although both treatments protect the brain from over-stimulation during severe stress (15, 24, 31) they likely employ different neuronal mechanisms. NPY is most probably involved in faster recovery from stress and participates in termination of CNS activity by interaction with specific NPY receptors as well as by restoration of endogenous NPY sources partly depleted by the stress (15, 32). In contrast, the melanocortin system via MC4 receptors plays an important role in stress integration. Inhibition of MC4 receptors localized in the paraventricular nucleus (PVN) of the hypothalamus are supposed to decrease activation of CRH producing neurons (33)

and attenuate the HPA response to psychological stress (Ryan et al., 2014). We further hypothesized that blockade of MC4 receptors localized in the locus coeruleus region of the brain can prevent stress triggered ACTH mediated elevation of catecholamine biosynthetic enzymes gene expression (34).

One of the common outcomes from both peptides used prior to SPS was absence of rise of GR in hippocampus (15, 24, 31) which could be beneficial for hippocampal regulation of the HPA axis and maintaining memory formation on a psychologically normal level. Long-term memories and cognition are an essential adaptive mechanism that allows an individual to effectively face similar demands again is impaired by traumatic stress [rev. in (35, 36)]. Severe stress triggers formation of strong long-term memories mediated by the activation of hippocampal GRs and coupled to the recruitment of the growth and pro-survival BDNF/cAMP response element-binding protein (CREB) pathway, which is well-known to be a general mechanism required for long-term memory formation [rev in (37)].

Although both NPY and HS014 were found to have a pronounced favorable effect against development of PTSD-like behavior in relatively high doses, it is important to obtain similar effects from as low dose as possible, which also might help to reduce any potential side effects. This study revealed that combining of NPY and HS014 in sub-threshold doses, which by themselves were unable to prevent the development of depressive-like behavior, were effective when infused prior to SPS. In this regards, anatomical and physiological evidence has shown different ways that NPY and α MSH can interact (20, 29). It has been demonstrated that within the hypothalamus GABA and NPY inhibit α MSH production in a dose-dependent manner (19), and chronic icv NPY infusion to rats decreased hypothalamic α MSH mRNA and protein (18). Conversely α MSH increased NPY release from hypothalamic slices (38) and icv administration of α MSH inhibited the anti-depressive effects of NPY on the Porsolt forced swim test (29). In contrast, blockade of MC4R with HS014 potentiated NPY action and promote a greater anti-depressive like behavior. A synergistic anxiolytic effect was also observed after direct administration of NPY and MC4R antagonist into the right amygdala where a dense population of NPY and α MSH containing fibers and abundance of NPY Y1 and MC4 receptors are found (20).

HS014 and NPY may also interact at the levels of signal transduction. The MC4R and receptors for NPY belong to the G-protein coupled receptor superfamily [rev in. (39, 40)].

Activation of MC4R increase cAMP levels (41) while NPY Y1 receptor stimulation result in inhibition of adenylyl cyclase activity (42), thus combined HS014 and NPY may synergistically lead to reduced cAMP.

5. Conclusion

Both MC4R antagonist and NPY, alone or combined, have potential for prophylactic treatment against traumatic stress triggered anxiety or depressive-like behaviors. NPY has more widespread potential for early intervention.

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Figure legends.

Figure 1. Effect of intranasal infusion of NPY or HS014 on immobility time during forced swim stress part of SPS stressors immediately after the immobilization. **A.** The scheme of the experiment is displayed. **B.** The data are expressed relative to vehicle treated group taken as 1 for each interval. The percentage of the total time spent immobile during 5 min intervals is shown as mean $\pm$ SEM. * $p \leq 0.05$, ** < 0.01 compared to vehicle (V) at the same time interval; ^a $p \leq 0.05$ compared to 3.5 ng HS014; ^b $p \leq 0.05$ compared to 100 μ g NPY. Results are pooled from two separate experiments, $n = 10$ per group.

Figure 2. Effect of intranasal infusion of NPY, HS014 or their combination on immobility time in forced swim test (FST) seven days after SPS stressors. The scheme of the experiment is displayed. The respective peptides were given by intranasal infusion 30 min prior to the SPS stressors. **A.** Effect of vehicle (V) 100 μ g NPY (100NPY), 3.5 ng and 100 μ g HS014 (3.5HS and 100HS) on immobility time. Data are expressed relative to unstressed control taken as 1 and shown as mean $\pm$ SEM. * $p \leq 0.05$, ** < 0.01 compared to control; [#] $p \leq 0.05$, ^{###} $p \leq 0.001$ compared to V; ^{\$\$} $p \leq 0.01$ compared to 3.5HS; ^{\$\$} $p \leq 0.01$ compared to 100NPY. **B.** Effect of 50 μ g NPY (50NPY), 3.5 ng HS014 (3.5HS) and 50 μ g NPY together with 3.5 ng HS014

(50NPY+3.5HS) on immobility time. Data expressed relative to unstressed control taken as 1 and shown as mean $\pm$ SEM. ** ≤ 0.01 compared to control; # $p \leq 0.05$, ## $p \leq 0.01$ compared to 50NPY or 3.5HS. n = 9 - 10 per group.

Figure 3. Effect of intranasal infusion of NPY and HS014 on TH mRNA levels in the locus coeruleus (A) and plasma corticosterone (B) and ACTH (C) levels 30 min after termination of SPS stressors. Data are expressed relative to vehicle treated group (V) taken as 1 and presented as mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$ compared to V group. Results are pooled from two separate experiments, n = 8-10 per group.

Figure 4. Effect of intranasal infusion of NPY or HS014 on GR protein in the hippocampus. Representative western blots and summary data are shown. Data are expressed relative to control group taken as 1 and presented as mean $\pm$ SEM. * $p \leq 0.05$ compared to control; # $p \leq 0.05$ compared to V group. Results are pooled from two separate experiments, n = 8-10 per group.

Figure 5. Effect of post-SPS intranasal infusion of HS014 on SPS-elicited anxiety-like behavior on EPM. Anxiety-like behavior was evaluated 7 days after SPS stressors in EPM for percentage of entries and time in open arm (OA), frequency and duration of risk assessment and defecation. Data are presented as mean $\pm$ SEM. ** $p \leq 0.01$, *** $p \leq 0.001$ compared to control; ## $p \leq 0.01$ compared to vehicle treated group (SPS/V); \$\$ $p \leq 0.01$ compared to SPS/3.5HS group, n=12.

Figure 6. Effect of post-SPS intranasal infusion of NPY and HS014 on immobility time in forced swim test following seven days after SPS stressors. *** $p \leq 0.001$ compared to control; # $p \leq 0.05$ ## $p < 0.01$ compared to vehicle treated group (SPS/V). n = 10 per group.

References

1. Kearns MC, Ressler KJ, Zatzick D, Rothbaum BO (2012): Early interventions for PTSD: a review. *Depress Anxiety*. 29:833-842.

2. Sones HM, Thorp SR, Raskind M (2011): Prevention of posttraumatic stress disorder. *Psychiatr Clin North Am.* 34:79-94.
3. Cohen H, Matar MA, Buskila D, Kaplan Z, Zohar J (2008): Early post-stressor intervention with high-dose corticosterone attenuates posttraumatic stress response in an animal model of posttraumatic stress disorder. *Biol Psychiatry.* 64:708-717.
4. Zohar J, Yahalom H, Kozlovsky N, Cwikel-Hamzany S, Matar MA, Kaplan Z, et al. (2011): High dose hydrocortisone immediately after trauma may alter the trajectory of PTSD: interplay between clinical and animal studies. *Eur Neuropsychopharmacol.* 21:796-809.
5. Gamber KM, Macarthur H, Westfall TC (2005): Cannabinoids augment the release of neuropeptide Y in the rat hypothalamus. *Neuropharmacology.* 49:646-652.
6. Adamec R, Fougere D, Risbrough V (2010): CRF receptor blockade prevents initiation and consolidation of stress effects on affect in the predator stress model of PTSD. *Int J Neuropsychopharmacol.* 13:747-757.
7. Kormos V, Gaszner B (2013): Role of neuropeptides in anxiety, stress, and depression: from animals to humans. *Neuropeptides.* 47:401-419.
8. Caruso V, Lagerstrom MC, Olszewski PK, Fredriksson R, Schioth HB (2014): Synaptic changes induced by melanocortin signalling. *Nat Rev Neurosci.* 15:98-110.
9. Paschos KA, Veletza S, Chatzaki E (2009): Neuropeptide and sigma receptors as novel therapeutic targets for the pharmacotherapy of depression. *CNS Drugs.* 23:755-772.
10. Eaton K, Sallee FR, Sah R (2007): Relevance of neuropeptide Y (NPY) in psychiatry. *Curr Top Med Chem.* 7:1645-1659.
11. Sah R, Geraciotti TD (2013): Neuropeptide y and posttraumatic stress disorder. *Molecular Psychiatry.* 18:646-655.
12. Rasmusson AM, Schnurr PP, Zukowska Z, Scioli E, Forman DE (2010): Adaptation to extreme stress: post-traumatic stress disorder, neuropeptide Y and metabolic syndrome. *Exp Biol Med (Maywood).* 235:1150-1162.
13. Pierce ME, Pritchard LM (2013): Hypothalamic-pituitary-adrenal axis dysregulation in posttraumatic stress disorder. *Psychology of Trauma: Nova Science Publishers, Inc.*, pp 225-236.
14. O'Donnell T, Hegadoren KM, Coupland NC (2004): Noradrenergic mechanisms in the pathophysiology of post-traumatic stress disorder. *Neuropsychobiology.* 50:273-283.
15. Serova LI, Tillinger A, Alaluf LG, Laukova M, Keegan K, Sabban EL (2013): Single intranasal neuropeptide Y infusion attenuates development of PTSD-like symptoms to traumatic stress in rats. *Neuroscience.* 236:298-312.
16. Serova LI, Laukova M, Alaluf LG, Pucillo L, Sabban EL (2014): Intranasal neuropeptide Y reverses anxiety and depressive-like behavior impaired by single prolonged stress PTSD model. *Eur Neuropsychopharmacol.* 24:142-147.
17. Serova LI, Laukova M, Alaluf LG, Sabban EL (2013): Intranasal infusion of melanocortin receptor four (MC4R) antagonist to rats ameliorates development of depression and anxiety related symptoms induced by single prolonged stress. *Behav Brain Res.* 250:139-147.
18. Blasquez C, Jégou S, Friard O, Tonon MC, Fournier A, Vaudry H (1995): Effect of centrally administered neuropeptide Y on hypothalamic and hypophyseal proopiomelanocortin-derived peptides in the rat. *Neuroscience.* 68:221-227.

19. Blasquez C, Jégou S, Tranchand Bunel D, Fournier A, Vaudry H (1992): Neuropeptide Y inhibits alpha-MSH release from rat hypothalamic slices through a pertussis toxin-sensitive G protein. *Brain Res.* 596:163-168.
20. Kokare DM, Dandekar MP, Chopde CT, Subhedar N (2005): Interaction between neuropeptide Y and alpha-melanocyte stimulating hormone in amygdala regulates anxiety in rats. *Brain Res.* 1043:107-114.
21. Khan S, Liberzon I (2004): Topiramate attenuates exaggerated acoustic startle in an animal model of PTSD. *Psychopharmacology (Berl).* 172:225-229.
22. Liberzon I, Sripada CS (2008): The functional neuroanatomy of PTSD: a critical review. *Prog Brain Res.* 167:151-169.
23. Yamamoto S, Morinobu S, Takei S, Fuchikami M, Matsuki A, Yamawaki S, et al. (2009): Single prolonged stress: toward an animal model of posttraumatic stress disorder. *Depress Anxiety.* 26:1110-1117.
24. Serova LI, Laukova M, Alaluf LG, Sabban EL (2014): Blockage of melanocortin 4 receptors by intranasal HS014 attenuates single prolonged stress triggered changes in several brain regions. *J Neurochemistry, in press.*
25. Pervanidou P, Chrousos GP (2010): Neuroendocrinology of post-traumatic stress disorder. *Prog Brain Res.* 182:149-160.
26. Yehuda R (2009): Status of glucocorticoid alterations in post-traumatic stress disorder. *Ann N Y Acad Sci.* 1179:56-69.
27. Olson VG, Rockett HR, Reh RK, Redila VA, Tran PM, Venkov HA, et al. (2011): The role of norepinephrine in differential response to stress in an animal model of posttraumatic stress disorder. *Biol Psychiatry.* 70:441-448.
28. Drossopoulou G, Antoniou K, Kitraki E, Papathanasiou G, Papalexi E, Dalla C, et al. (2004): Sex differences in behavioral, neurochemical and neuroendocrine effects induced by the forced swim test in rats. *Neuroscience.* 126:849-857.
29. Goyal SN, Kokare DM, Chopde CT, Subhedar NK (2006): Alpha-melanocyte stimulating hormone antagonizes antidepressant-like effect of neuropeptide Y in Porsolt's test in rats. *Pharmacol Biochem Behav.* 85:369-377.
30. Page ME, Detke MJ, Dalvi A, Kirby LG, Lucki I (1999): Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology (Berl).* 147:162-167.
31. Laukova M, Alaluf LG, Serova LI, Arango V, Sabban EL (2014): Early Intervention With Intranasal NPY Prevents Single Prolonged Stress-Triggered Impairments in Hypothalamus and Ventral Hippocampus in Male Rats. *Endocrinology.* 155:3920-3933.
32. Kash TL, Winder DG (2006): Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis. *Neuropharmacology.* 51:1013-1022.
33. Maniam J, Morris MJ (2012): The link between stress and feeding behaviour. *Neuropharmacology.* 63:97-110.
34. Serova LI, Gueorguiev V, Cheng SY, Sabban EL (2008): Adrenocorticotrophic hormone elevates gene expression for catecholamine biosynthesis in rat superior cervical ganglia and locus coeruleus by an adrenal independent mechanism. *Neuroscience.* 153:1380-1389.
35. Johnson LR, McGuire J, Lazarus R, Palmer AA (2012): Pavlovian fear memory circuits and phenotypic models of PTSD. *Neuropharmacology.* 62:638-646.

36. Johansen JP, Cain CK, Ostroff LE, LeDoux JE (2011): Molecular mechanisms of fear learning and memory. *Cell*. 147:509-524.
37. Finsterwald C, Alberini CM (2014): Stress and glucocorticoid receptor-dependent mechanisms in long-term memory: from adaptive responses to psychopathologies. *Neurobiol Learn Mem*. 112:17-29.
38. King PJ, Widdowson PS, Doods H, Williams G (2000): Regulation of neuropeptide Y release from hypothalamic slices by melanocortin-4 agonists and leptin. *Peptides*. 21:45-48.
39. Getting S (2006): Targeting melanocortin receptors as potential novel therapeutics. *Pharmacology & Therapeutics*. 111:1-15.
40. Eva C, Serra M, Mele P, Panzica G, Oberto A (2006): Physiology and gene regulation of the brain NPY Y1 receptor. *Front Neuroendocrinol*. 27:308-339.
41. Hadley ME, Haskell-Luevano C (1999): The proopiomelanocortin system. *Ann N Y Acad Sci*. 885:1-21.
42. Herzog H, Hort YJ, Ball HJ, Hayes G, Shine J, Selbie LA (1992): Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc Natl Acad Sci U S A*. 89:5794-5798.

Fig. 1

A

B

Fig. 2

Prior to SPS treatments

Fig. 3

Prior to SPS treatments

Fig. 4

Hippocampus
Glucocorticoid receptors (GR)

Fig. 5 Post- SPS treatments

Risk assessment

Defecation

Fig. 6

Post- SPS treatments

Table 1. Effect of intranasal infusion of NPY and HS014 prior to SPS on anxiety-like behavior on EPM compared to vehicle infused rats.

EPM	HS014	NPY
Entries (%): in OA	increased*	increased*
in CA	decreased, 3.5ng*	decreased*
Duration(%): in OA	increased *	increased*
in CA	decreased*	no effect
Anxiety index	decreased*	decreased**
Risk assessment:		
frequency	increased *	increased*
duration	increased*	increased**
Grooming duration	decreased, 100 µg*	no effect

*p < 0.05, **p < 0.01 calculated by Tukey's Multiple Comparison Test.