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Preclinical Studies on Intranasal NPY and Selective NPY Receptor Agonists in Rodent PTSD Model: Focus on Locus Coeruleus Noradrenergic System

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Preclinical Studies on Intranasal NPY and Selective NPY Receptor Agonists in Rodent PTSD Model: Focus on Locus Coeruleus Noradrenergic System

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This dissertation is dedicated to my parents, for their love, support, inspiration and encouragement through the years.

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ABBREVIATIONS

ACTH: Adrenocorticotropic hormone

ADHD: Attention deficit hyperactivity disorder

ASR: Acoustic startle response

BBB: Blood brain barrier

BLA: Basolateral amygdala

CA: Closed arm

CRH: Corticotropin-releasing hormone

CRHR1: Corticotropin-releasing hormone receptor 1

CNS: Central Nervous System

CRF: Corticotropin-releasing factor

CSF: Cerebrospinal fluid

DAT: Dopamine transporter

EPM: Elevated plus maze

FST: Forced swim test

FKBP5: FK506 binding protein 5

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GR: Glucocorticoid receptor

HPA: Hypothalamic-pituitary-adrenocortical axis

ICV: Intracerebroventricular injection

KO: Knock out

LC-NE: Locus coeruleus/norepinephrine

LC: Locus coeruleus

MR: Mineralocorticoids

NE: Noerpinephrine

NET: Norepinephrine transporter

NPY: Neuropeptide Y

OA: Open arm

PTSD: posttraumatic stress disorder

PVN: Paraventricular nucleus

SI: Social interaction

SLC6A2: Solute Carrier Family 6 Member 2

SPS: Single Prolonged stress

SSRI: Selective serotonin reuptake inhibitor

TSS: Transcription start site

Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychological disorder that develops in a subset of individuals after exposure to a traumatic stress. Neuropeptide Y (NPY) an endogenous 36 amino acid neuropeptide expressed in many areas of the brain and periphery especially those involved in stress may confer resilience to the harmful effect of stress.

We demonstrated changes in the Single Prolonged Stress (SPS) PTSD model and potential for their modulation by delivery of intranasal NPY to the brain. There was a progressive worsening of anxiety symptoms at two weeks compared to one week after SPS. Previously we showed that 150µg of NPY was effective to reverse anxiety, depressive-like and hyperarousal symptoms one week after SPS, however this dose was not sufficient to reverse anxiety symptoms two weeks after SPS when symptoms have become more severe. We determined that doubling NPY dose to 300µg was sufficient to reverse symptoms of anxiety, depression and hyperarousal two weeks after more severe symptoms have manifested.

Activation of the NPY Y1R subtype was sufficient to prevent the development of SPS elicited anxiety, social impairment and depressive-like behavior. Moreover, intranasal delivery of [D-His^{26}] NPY was superior to NPY for preventing depressive-like behavior and has promise as an early intervention therapy following traumatic stress. Significant molecular impairments in gene expression for corticotrophin releasing hormone (CRH) and neuropeptide Y (NPY) systems in the locus coeruleus (LC) and mediobasal hypothalamus were observed two weeks following SPS. There was a divergence in the expression levels of the norepinephrine transporter (NET) mRNA in the LC after SPS, some animals had significantly higher NET mRNA levels and some had levels were

similar or lower than unstressed controls. Nevertheless, NET protein levels were reduced in the hippocampus; projection region, likely by increasing noradrenergic activation. The SPS triggered hyperarousal was associated with the changes in NET gene expression. Animals with increased startle response also had increased NET mRNA. Within the subgroup of animals that had a lower acoustic startle response, there was a significant negative correlation with NET mRNA expression. Furthermore, SPS showed a potential effect in the epigenetic regulation of the NET. Increased methylation of the NET gene promoter region was observed in the lower NET responsive group and was associated with a reduction in NET mRNA expression.

Overall these results shed new insights into mechanisms for resilience or susceptibility to development of hyperarousal, a diagnostic feature of PTSD and that NPY or a specific Y1R agonist (D-His^{26})NPY can effectively treat core symptoms of PTSD in an animal model.

Introduction

Post-Traumatic Stress Disorder (PTSD)

Post-traumatic stress disorder (PTSD) is a debilitating neuropsychiatric disorder that affects a subset of individuals exposed to severe or life-threatening traumatic stress. PTSD is characterized (DSM-5) by persistent symptoms for at least a month after the trauma: which include: intrusive memories such as flashbacks or nightmares; avoidance of stimuli associated with the trauma; hyperarousal or hypervigilance state; negative alterations of cognition and mood associated with the traumatic event.

Burdens associated with PTSD and its comorbidities are extremely severe. PTSD is comorbid with anxiety disorders, substance abuse, sleep disturbances and worsening medical conditions [1-3]. Individuals with PTSD have a high risk for suicide and indeed PTSD related suicide is the 10th leading cause of death in the United States [4]. The symptoms of PTSD can be very long lasting, with enormous personal and society cost. Some symptoms, such as cognitive abnormalities actually worsen with time [5]. The disorder can persist for more than 40 years [6]. Therefore, it is extremely important to understand the mechanisms of resilience or susceptibility to development of PTSD and to be able to increase resilience. Early childhood adversary, by way of epigenetic changes, is associated with increased susceptibility [7].

Treating PTSD can help to regain a sense of control over one's life. Treatments include psychotherapy and/or medications. Combining these treatments can help improve PTSD symptomology; however, the use of medications has not been very effective. For example, paroxetine and other selective serotonin reuptake inhibitors (SSRIs) are FDA approved medications for PTSD treatment but have not been sufficiently effective in

treating PTSD [8, 9] consequently resulting in a dire need for effective and more specific PTSD treatments.

AIMS

Aim 1A: Evaluate progression in the development of anxiety symptoms and gene expression changes in the mediobasal hypothalamus and the locus coeruleus (LC) in the single prolonged stress (SPS) animal model of PTSD.

Aim 1B: Assess whether Neuropeptide Y (NPY) can effectively reverse PTSD core symptoms in the PTSD animal model.

Aim 2: Determine the NPY receptor subtype that is sufficient to prevent development of anxiety and depressive like effects.

Aim 3: Evaluate gene expression changes in the LC and mediobasal hypothalamus and the role of norepinephrine transporter (NET) in hyperarousal symptoms

Engagement of the hypothalamic pituitary adrenal (HPA) axis and the LC/NE in post-Traumatic Stress Disorder (PTSD)

Post-traumatic stress disorder (PTSD) leads to the dysregulation of the hypothalamic pituitary adrenal (HPA) axis (Figure 1). Considerable clinical evidence also suggests the impairment in the locus coeruleus/norepinephrine-autonomic (LC/NE) noradrenergic system. [10-12]. The HPA neuroendocrine axis and the LC/NE nervous system are important parts of highly conserved stress regulatory systems and are involved in the development and manifestation of PTSD [13, 14].

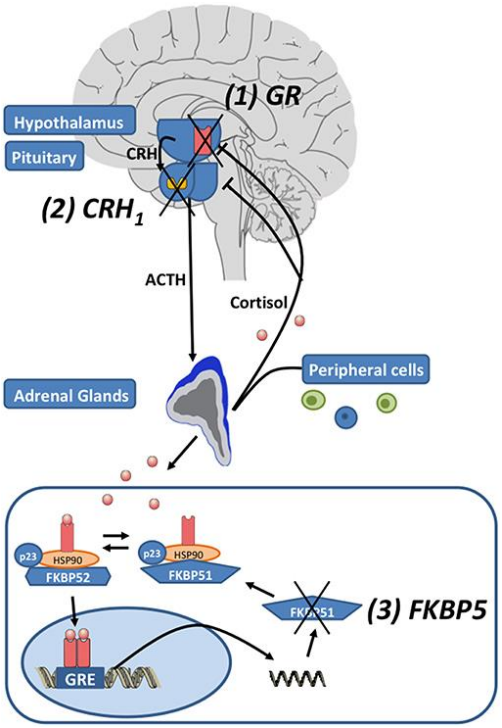
The HPA axis consists of direct and feedback interactions among the hypothalamus, the pituitary gland and the adrenal gland (Figure 1) comprising the neuroendocrine system controlling reactions to stress and regulates [15] many body processes. Activation of the HPA axis depends on type of stressor and duration. Stress drives the HPA stress response, and feedback mechanisms effectively terminate the response after the stressor becomes less intense or subsides. Corticotrophin releasing hormone (CRH), a principle regulator of the HPA axis is synthesized and secreted by neurons localized in the medial parvocellular subdivision of the paraventricular nucleus (PVN) [16, 17]. CRH is secreted from the PVN of the hypothalamus in response to stress. CRH stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) (Figure 1). The pituitary gland releases ACTH into the bloodstream. ACTH in the blood stream binds to receptors on the surface of the adrenal cortices eliciting intracellular events that result in the adrenal glands secreting glucocorticoids. Under normal physiological conditions, glucocorticoids increase glucose supply to skeletal muscles, providing the body with extra energy to respond to stressors. The normal functioning of the HPA axis is essential for coping with stress, but repeated or chronic stress has the potential to disrupt the beneficial physiological role of the HPA axis.

Glucocorticoids bind to mineral corticoid (MR) with higher affinity than they do to the glucocorticoid receptor (GR) [18, 19]. Both GR and MR operate in a complementary fashion to regulate HPA-axis. GR is expressed ubiquitously in the brain while MR is predominately expressed in limbic areas such as the hippocampus and amygdala [20-22]. Due to a higher affinity of glucocorticoids to MR, the MR has a higher occupancy rate in non-stressful conditions in order to maintain low basal corticosteroid levels through negative feedback [21]. However, full GR occupancy is only reached at peak cortisol concentrations such as during stress. Impairments in GR and HPA regulators such as FK506 binding protein 5 (FKBP5) have been associated with PTSD susceptibility [23].

GR plays an essential role in glucocorticoid feedback when it is activated by ligand, it represses CRH biosynthesis via a direct negative feedback on the hypothalamus which leads to the termination of the stress response. FKBP5 can modulate GR sensitivity to its ligand and FKBP5 mRNA and protein expression are induced by GR activation via intronic hormone response elements providing an ultra-short feedback loop for regulating GR-sensitivity [24].

Polymorphisms in the FKBP5 gene have been associated with differences in GR sensitivity, stress hormone system regulation and biologically distinct subtypes of PTSD and can predict the severity of its onset [25, 26].

Figure 1 - HPA axis



Adapted from Menke et al., Front. Psychiatry, 28 February 2019 [27].

Figure 1. Hypothalamic Pituitary Adrenal (HPA) axis regulation. Corticotrophin-releasing hormone (CRH) is released by neurons in the paraventricular nucleus of the hypothalamus (1). Subsequently CRH receptors are activated and the secretion of adrenocorticotropic hormone (ACTH) from the pituitary is induced (2). ACTH induces the release of glucocorticoids (cortisol) by the adrenal glands. After the activation of the HPA axis, negative feedback loops are activated to reinstate homeostasis by cortisol activating glucocorticoid receptors (GR). The unliganded GR complex consists of the co-chaperones FKBP51 or FKBP52 (encoded by their respective genes *FKBP5* and *FKBP4*), p23 (a co-chaperone molecule) and hsp90 dimer. When FKBP51 binds to the GR-complex via hsp90, the GR affinity for cortisol is reduced. When glucocorticoids bind to the GR, FKBP51 is exchanged against FKBP52 and the nuclear translocation of the ligand-bound GR is enabled. The GR directly binds to the DNA via glucocorticoid response elements

(GREs) and induces FKBP5 mRNA expression (3) and subsequently FKBP51 production, inducing an ultra-short negative feedback loop on GR sensitivity.

Locus coeruleus/norepinephrine-autonomic (LC/NE) noradrenergic system.

The LC is situated deep in the pons and sends projections to most brain regions, including the brainstem, the cerebellum, the diencephalon and the paleo- and neocortex CNS. It is comprised of neurons in rats and the main source of norepinephrine (NE) in the brain [28]. The actions of NE are very diverse suggesting that the LC is engaged in many functions. The LC-NE is one of the first systems to be engaged following a stressful event. LC neurons display increased activity in response to arousing stimuli but during quiet wakefulness neurons in the LC are less active and their activity diminishes during rapid eye movement, or REM, sleep. Physiological levels of norepinephrine in the prefrontal cortex, an area of the brain involved with attention, has been shown to be important in the facilitation of attention-related tasks. The LC/NE system is integral to a number of higher cognitive functions ranging from, fine-tuning sensory signals, to motivation and working memory.

Figure 2 - Major afferent and efferent projections of the LC

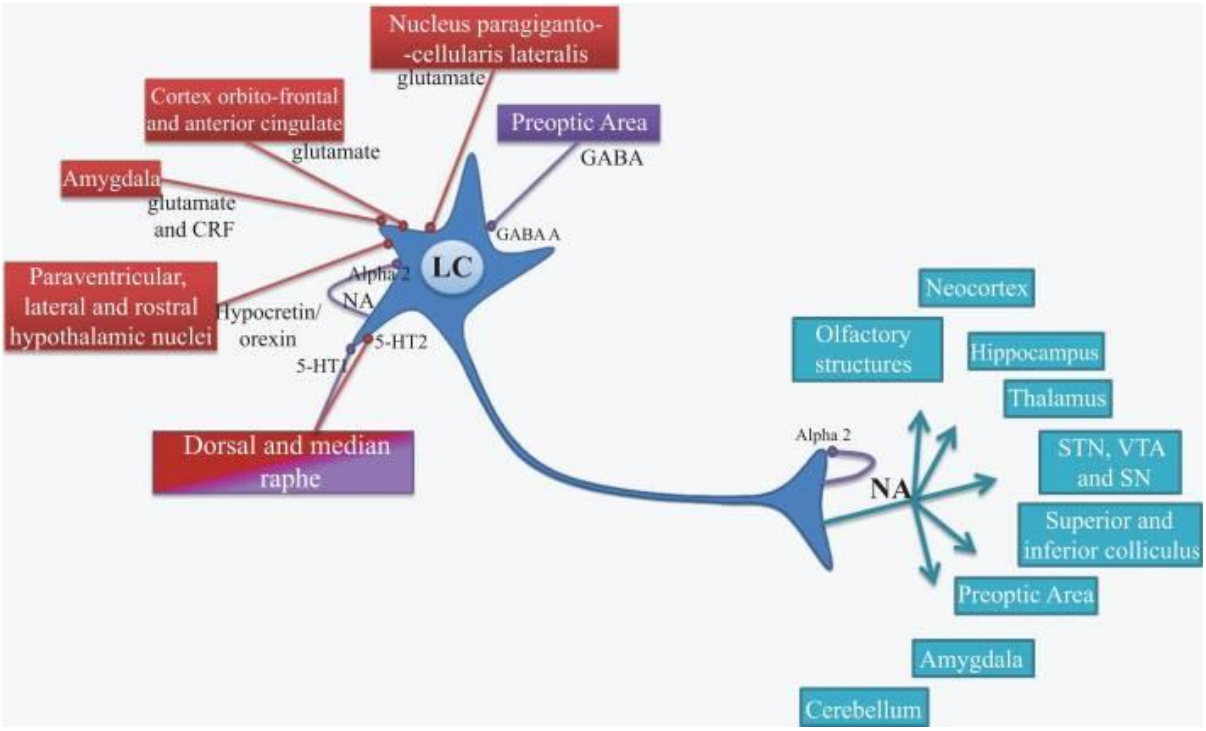


Figure 2. Afferent (red and purple) and efferent projections of LC neurons (blue). LC, locus coeruleus; NA, noradrenaline; Alpha2, Alpha2 receptor; GABA, γ-aminobutyric acid; GABA A, GABA A receptor; 5-HT, serotonin; 5-HT1 and 5-HT2, serotonin receptor subtype 1 and 2; CRF, corticotropin releasing factor; STN, subthalamic nucleus; VTA, ventral tegmental area; SN, substantia nigra; NET, noradrenalinergic transporters; Red, excitatory input; Purple, inhibitory input.

Adapted from Delaville et al., Frontiers in Systems Neuroscience, 2011[29].

Stress increases activity and subsequently gene expression of NE-producing enzymes, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) [30-32]. Increased tonic activity of the LC-NE system is necessary and sufficient for stress-induced anxiety and aversion [33]. Endogenous corticotropin releasing hormone (CRH) from the amygdala induces anxiety-like behaviors via its terminals in the LC and increases the tonic firing activity of LC neurons [33]. Optogenetics a method for controlling a neuron's activity using light and genetic engineering was used for stimulation of the LC. Norepinephrine release in the basolateral amygdala (BLA) increased anxiety-like behavior following photostimulation of LC-NE fibers [33]. The increased NE release in the BLA mediate an anxiety-like phenotype via β-adrenergic receptors [33]. Chronic and repeated stress leads to increased changes in signaling pathways and more sustained transcriptionally mediated induction of TH and DBH, yielding elevated NE levels in some terminal fields [31, 32, 34-36].

Elevated levels of NE in the CSF has been directly linked to severity of PTSD core symptoms in patients [37] and increased LC activity has been linked to hyperarousal and re-experiencing features of PTSD [38]. The LC-hippocampal network is involved in the formation of specific memories. LC neurons originally defined by their canonical norepinephrine noradrenaline NE) signaling, mediate post encoding novelty-associated enhancement of memory retention in a manner consistent with possible co-release of DA along with NE in the hippocampus [39]. A memory is formed immediately upon experience and can last up to a lifetime. Input from LC to CA3 is essential for the formation of a persistent memory in the hippocampus [40]. Elevating noradrenergic activity at alpha-1-receptors (α-1-ARs) in the medial prefrontal cortex also facilitates cognitive performance of rats in an attentional set-shifting task, a measure of attention and cognitive flexibility in rats [41]. However, overstimulation of medial prefrontal cortex (mPFC) projecting LC cells disrupted the memory extinction process through activation of α-1-

ARs in mPFC [42]. Several groups have demonstrated that LC-NE neurons are activated by many different stressors. Selective inhibition of LC-NE neurons during stress prevents subsequent anxiety-like behavior. Exogenously increasing tonic activity of LC-NE neurons is alone sufficient for anxiety-like and aversive behavior [33]. McCall et al. also found that endogenous corticotropin-releasing hormone(+) (CRH(+)) LC inputs from the amygdala increase tonic LC activity, inducing anxiety-like behaviors [33]. Based on these studies the LC-NE system is a critical mediator of stress-induced anxiety and critical target for therapeutic intervention to prevent stress-related affective disorders.

Norepinephrine transporter (NET)

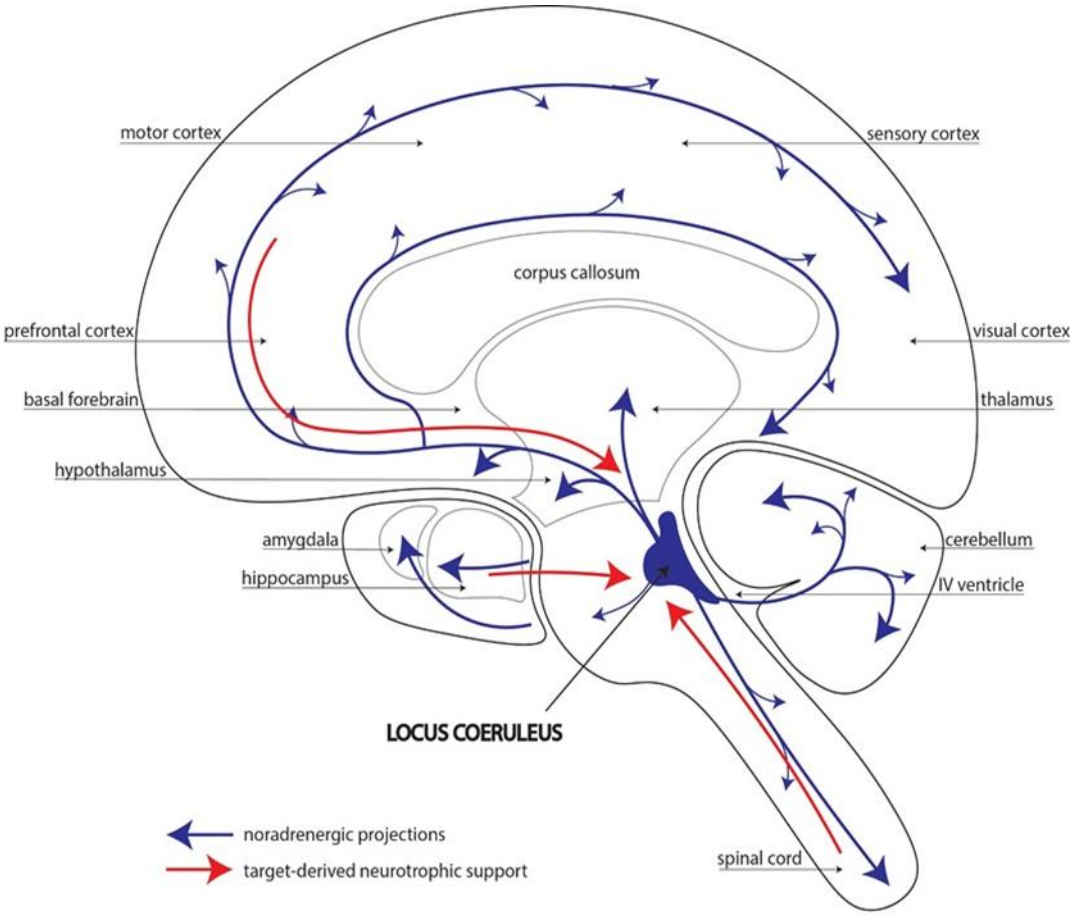
The LC-NE signaling is controlled to a large extent by norepinephrine transporters (NET) by mediating the rapid clearance of NE from the synaptic cleft and maintaining NE storage in the pre-synaptic noradrenergic cells (Figure 4) [43, 44]. NET belongs to the family of sodium chloride neurotransmitter transporters [45, 46] with its concentration highest in the LC and can be found in lower concentrations in the cortical and subcortical regions, including the frontal cortex, hippocampus, amygdala, thalamus, and cerebellar cortex [47]. NET knockout mice have a lower rate of extracellular NE clearance in the brain and elevated extracellular NE concentrations in plasma as well as altered dopamine signaling [48, 49]. They also display higher resistance to convulsions [50]. NET are targets for the action of many drugs used to treat major depression [51]. Stimulant drugs that act on both NET and DA transporters (DAT) are used to treat attention deficit/hyperactivity disorder (ADHD), however a NET selective drug (reboxetine) has been shown to have good efficacy in treating ADHD [52]. In addition, the engagement of NE systems in response to stress suggest that NE is implicated in disorders triggered by trauma events such as in post-traumatic stress disorder and depression [53] and noradrenergic activation has been shown to

significantly affect the maintenance of arousal, an important cognitive function impaired in ADHD.

Furthermore, psychiatric and cardiovascular phenotypes have resulted from nonsynonymous single nucleotide polymorphisms (SNPs) leading to amino acid substitutions in hNET [54-57]. NET availability in the locus coeruleus and limbic brain regions of PTSD patients was decreased compared to healthy controls [58]. Characterizing NET availability following the healthy human adaptation of stress and the development of PTSD would shed light on noradrenergic contributions to the human stress response. More recently DNA methylation has received increased attention as a possible modulator in psychiatric disorders in addition to the influence of genetic polymorphisms.

DNA methylation is a biological process by which methyl groups are added to the DNA molecule at CpG sites, which can change the activity of a DNA segment without changing the sequence. If methylation occurs at the gene promoter, the gene transcription.is typically repressed. CpG sites are regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction. A study in veterans showed that low methylation level in a specific NET promoter region may independently have higher risk of PTSD (Jingmei Zhang, OAT 2016). Another study showed that NET promoter hypermethylation was detected toward the 5′ end of the promoter in ADHD patients compared to healthy controls. There was a negative association between hyperactivity–impulsivity symptom scores with NET methylation levels for several CpG sites [59]. In addition, there was a negative correlation between methylation of a single CpG site with in vivo NET expression in several brain regions in ADHD patients [59]. The differential methylation seen in these patients may be due to transcription factors behaving in a distinct manner in ADHD. These studies reveal the potential effects of epigenetics on behavioral control in health and disease states.

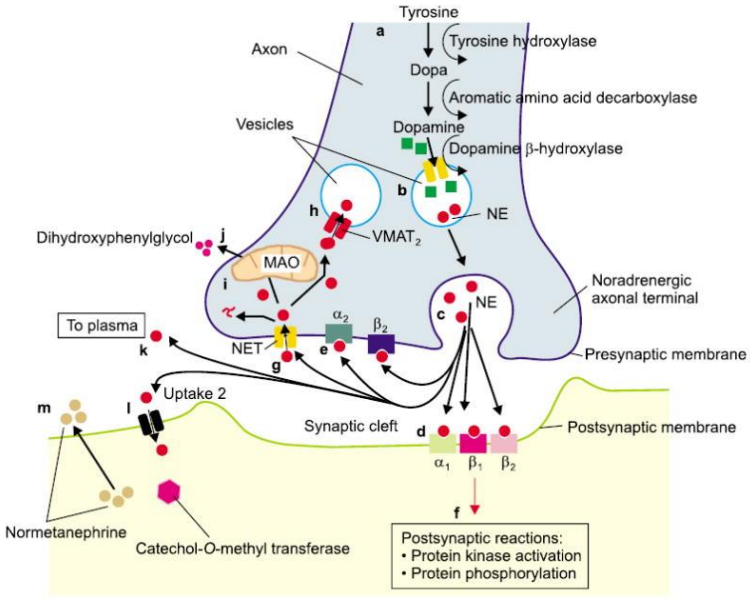
Figure 3 – LC/NE Noradrenergic System



Adapted from Feinstein et al., Journal of Neurochemistry, 2016 [60].

Figure 3. Locus coeruleus neurons sends projections to virtually every region of the CNS (blue arrows). In addition, it is the only source of NA to areas of the forebrain and cerebellum. Maintenance of noradrenergic innervation at some regions, most notably the forebrain and spinal cord, depends largely upon target-derived neurotrophin signaling (red arrows).

Figure 4 - Release and reuptake of norepinephrine (NE)



Adapted from Zhou et al., Drugs Future [61].

Figure 4. Diagram of a noradrenergic axonal terminal showing the release and reuptake of norepinephrine (NE). a. NE is synthesized from tyrosine via hydroxylation to form dihydroxyphenylalanine (Dopa), decarboxylation to form dopamine, and hydroxylation to form NE, and b stored in vesicles. c. As a result of an appropriate stimulus (not shown), NE is released into the synaptic cleft. d. Released NE activates the adrenergic receptors located on the postsynaptic membrane (α_1, β_1 and β) and the e. presynaptic membrane (α_2 and β_2) and causes f. postsynaptic reactions such as protein kinase activation and protein phosphorylation. g. The NET is responsible for reuptake of NE in the synaptic cleft and terminates its action. h. After reuptake by the NET, a small portion of the NE is restored in vesicles (following uptake by the vesicular amine transporter 2, $VMAT_2$); I. the rest is metabolized in the mitochondria by the enzyme monoamine oxidase (MAO), and j. the product dihydroxyphenylglycol (DHPG) is released into the circulation. k. A small portion

of the synaptic NE leaks into the circulation, or l. is taken up by another system (uptake 2) and m. metabolized to form normetanephrine (NMN).

Neuropeptide Y (NPY)

NPY is a 36 amino acid C-terminal amidated neuropeptide (see Fig 5) expressed abundantly in the central and peripheral nervous system [62-64]. In the brain, it is synthesized primarily by cell bodies in the arcuate nucleus of the hypothalamus and transported to the PVN where the highest concentrations are found. NPY is highly conserved among species and involved in the regulation of many systems throughout the body such as sleep, appetite, memory, anxiety, fear, and stress [65-67]. NPY is also expressed in sympathetic nerves and the adrenal medulla. In sympathetic excitation NPY is co-expressed with NE and enhances the vasoconstrictor effect of NE as well as that of angiotensin II [68, 69]. NPY immunoreactive fibers are present in high density in the mammalian heart and vicinity of blood vessels and play a role in the excitation-contraction (EC) coupling in cardiomyocytes and vascular smooth muscle cells.

It is synthesized in GABAergic neurons and expressed mainly in interneurons [63]. High levels of NPY are found in the hypothalamus, the periaqueductal gray, the septum, and the locus coeruleus (LC) and the nucleus accumbens [70, 71]. Moderate levels of NPY are found in the cerebral cortex, amygdala, the basal ganglia, hippocampus and the thalamus [62, 71]. In the NPY KO animals, mice with both mutant alleles for NPY do not make detectable NPY messenger RNA in brain or show NPY immunoreactivity in the brain or adrenal gland. However, a subset of young adult NPY KO mice had mild seizures, suggesting an inhibitory role of NPY on neural excitability during development [72]. Increased anxiety-like behavior [73] has been observed in NPY deficient mice. Transgenic

overexpression or intracerebroventricular (icv) administration of NPY has been shown to be anxiolytic and anti-depressive in rats and mice [74-77].

These studies suggest that Neuropeptide Y (NPY) expression is linked with the development of stress resilience in rodents.

Furthermore, the Sabban laboratory has shown that delivery of NPY to the CNS in rats by intranasal infusion is effective in preventing development of core PTSD symptoms, such as anxiety, hyperarousal and depressive-like behavior [78-82]. Intranasal delivery of NPY after manifestation of some of the PTSD core symptoms reversed the PTSD-associated symptoms. In both preclinical and clinical models, NPY levels are associated with resilience to harmful effects of stress [80, 83-90]. Patients with depression and anxiety disorders have abnormally low levels of NPY in plasma and cerebrospinal fluid (CSF) [85, 91-94]. PTSD patients showed a dose-dependent anxiolytic effect of intranasal NPY in a clinical trial [95].

Overall, pre-clinical and clinical studies show positive correlation of NPY with resilience to stress thus making NPY a potential therapy for some PTSD symptoms [78, 86, 95-97].

Figure 5 - Structure of Neuropeptide Y (NPY)

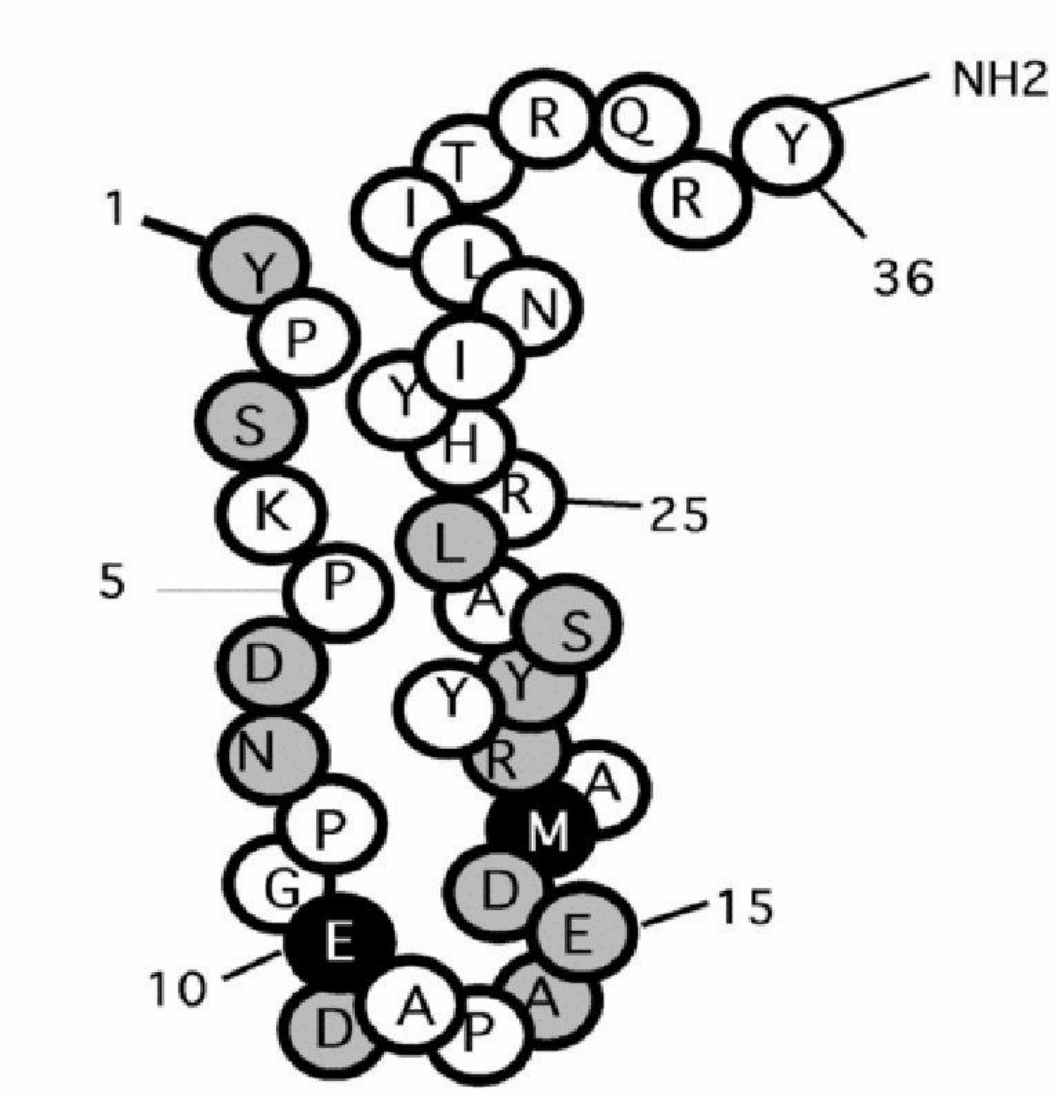


Figure 5. NPY is a 36 amino-acid neuropeptide with COOH-terminally alpha-amidated amino acid.

Adapted from Orbetzova et al., Appetite Regulatory Peptides, 2012 [98].

NPY receptors

NPY exerts its effects by binding G-protein coupled receptor proteins, Y1R, Y2R, Y4R and Y5R receptor subtypes with high affinity while Peptide YY, a short peptide released from cells in the ileum and colon in response to feeding, is the preferred agonist for the Y4R [65]. Y6R has also been cloned however, it is active only in rabbits and mice [99]. NPY receptors are coupled via Gi/o to several downstream pathways including inhibition of adenylyl cyclase, regulation of intracellular Ca2+, activation of MAPK, inward rectifying potassium channels, hyperpolarization-activated cation currents (lh) channel [89, 100].

N- and C-terminal of NPY are essential for NPY binding and activity of Y1R. Analogues with N-terminal truncations become less specific for the Y1R. The truncation of the first amino acid NPY(2–36) results in a loss of affinity [101]. The C-terminal contains an amide group and truncations show the importance of the amide group in the binding with the receptor [102]. Positions 7, 25, 26, 31, and 34 are important for subtype selectivity (Figure 7). [D-Arg^{25}]NPY and [D-His^{26}]NPY bind selectively to the Y1 receptor when substitutions at positions 25 and 26 are made (Figure 7) [103]. The affinity of the peptide to Y1/Y5 receptors was achieved with Pro in position 34. Introducing an aromatic amino acid e.g., Phenylalanine (Phe) at position 7 gives rise to a selective Y1 receptor binder [Phe^{7}, Pro^{34}]pNPY. In addition combining Pro34 with an exchange in position 31 by Leu contributes to aY1/Y4/Y5 receptor selectivity [104-106].

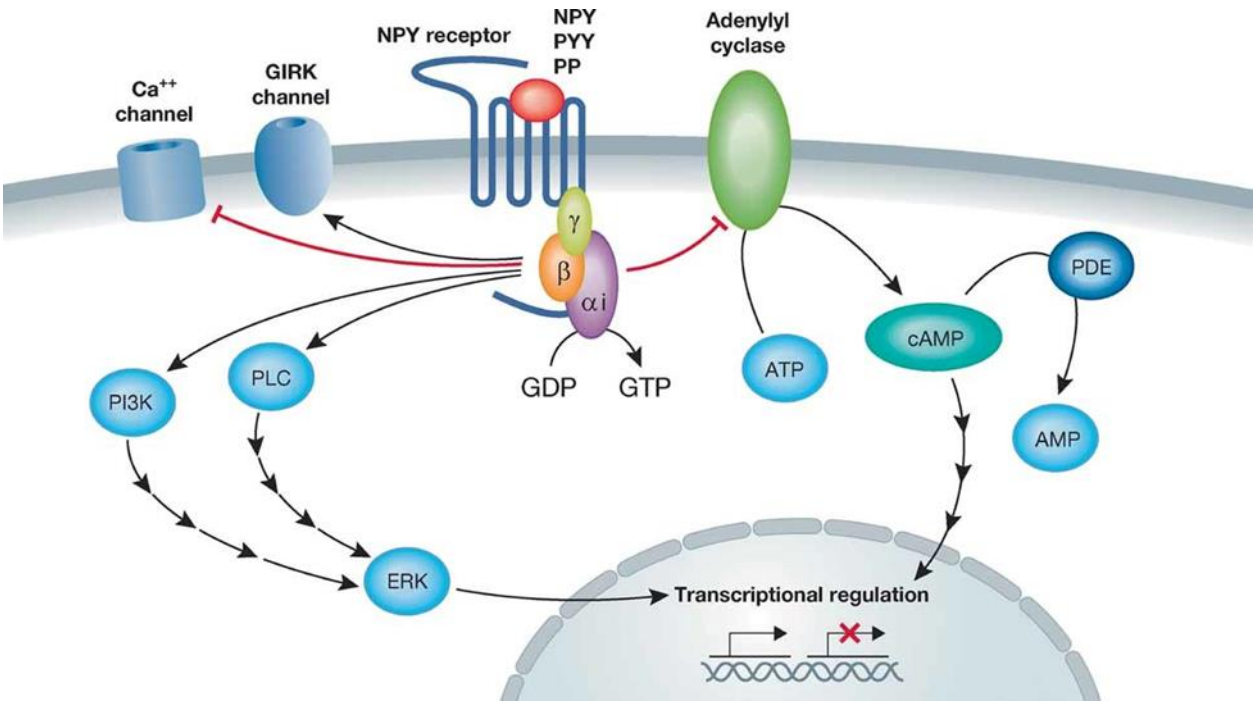
The Y1R was the first NPY receptor cloned [107-110] and is well conserved throughout evolution in mammalian and non-mammalian species. Both the N- and C– terminus are necessary for NPY to bind to Y1R [101, 102, 111].

Many of the anti-stress effects of NPY were proposed to be mediated by post synaptic Y1R or require Y1R, as shown by pharmacological and genetic approaches in naïve (not previously stressed) animals. For example, [Leu^{31}Pro^{34}]PYY, a Y1R/Y5R agonist, reduced immobility in Forced Swim Test (FST), while a Y2R agonist was ineffective [112, 113]. The anxiolytic effects of NPY generally is proposed to involve the Y1 receptor subtype [85, 114-118] although Y2 and Y5 receptors have also been implicated [85, 115, 116]. The icv injection of specific Y1 receptor agonist [D-His^{26}]NPY reduced basal anxiety 1 h later [119].

Emerging evidence also suggests an important role for Y2R. NPY knockout (KO) in mice have increased anxiety and dramatically enhanced acquisition of conditioned fear. Y1R and Y2R receptor single KO mice exhibited moderate changes in fear processing while the phenotype of NPY KO mice was fully recapitulated when both Y1R and Y2R were deleted [120]. Both Y1R agonists and Y2R antagonists induce antidepressant-like effects in mice [121]. Thus, it was proposed that Y2R has a synergistic role with Y1R in fear acquisition and extinction [122]. Other NPY receptor studies also show that Y4R preferentially binds pancreatic polypeptide (PP) compared to PYY and NPY. Y4R protein is expressed in few brain regions including the medial pre-optic area, the paraventricular hypothalamic nucleus, interpeduncular nuclei and the area postrema [123-125].

The NPY Y5 receptor has been cloned from human, rat [126, 127] mouse and dog. NPY Y_5 receptor immunoreactivity, binding sites and NPY Y5 receptor mRNA has been reported in several brain structures, including the limbic system and the brainstem. NPY Y5 receptor agonist have anxiolytic effects. Y5 receptor antagonists are anxiogenic in rats [115, 119, 128].

Figure 6 - Representative G-protein Therapeutic potential of neuropeptide Y (NPY) receptor ligands



Adapted from Wahlestedt et al., EMBO Mol Med 2010 [65].

Figure 6. NPY receptors couple to the Gi signaling cascade. Agonist binding activates the alpha subunit, which inactivates adenylyl cyclase. The beta/gamma subunit activates a number of different kinase cascades. Activation of the G protein complex can also lead to depressed Ca++ channel activity and enhanced G protein coupled inwardly rectifying potassium (GIRK) currents. These cellular signaling cascades have effects on initiation of gene transcription, and physiologic effects such as the stimulation or inhibition of hormone/neurotransmitter release.

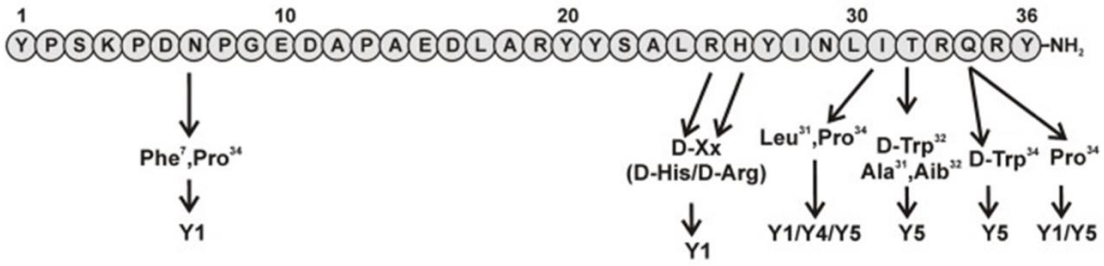
Table 1. Relative distribution of NPY receptors in the CNS

(++++: very high; +++: high; ++: moderate; +: low; +/−: very low; −: undetectable.

Y_1	Y_2	Y_4	Y_5	
Frontal cortex	++++	+	−	+/−
Lateral septum	+	++++	−	+/−
Lateral dorsal septum	++	++++	−	+
Nucleus accumbens	+	++	−	−
Bed nucleus of stria terminalis	+	++	−	+/−
Medial pre-optic area	+	++	++	+/−
Paraventricular nucleus	+	++	+++	+
Lateral hypothalamus	+	++	−	−
Perifornical area	+	++	−	+/−
Arcuate nucleus	+	++	−	−
Amygdala	++	++	−	+/−
Dorsal hippocampus	+/++	++/+++	+/−	+
Ventral tegmental area	+	++	−	+/−
Central gray	+	+	−	−
Dorsal raphe	+/−	++	−	−
LC	+/−	+	+/−	+/−
Pontine tegmental nucleus	+/−	++	+/−	+/−
Nucleus of the solitary tract	+++	++	+++	++
Area postrema	++	++++	++++	+

Adapted from Kask et al., Reviews, 2002 [63].

Figure 7 - Neuropeptide Y receptors subtype selectivity



Adapted from Pedragosa-Badia X et al., Frontiers Endocrinology, 2013 [129].

Figure 7. Important amino acid positions and truncated peptides to introduce selectivity to NPY receptors.

Intranasal delivery of drugs to the brain

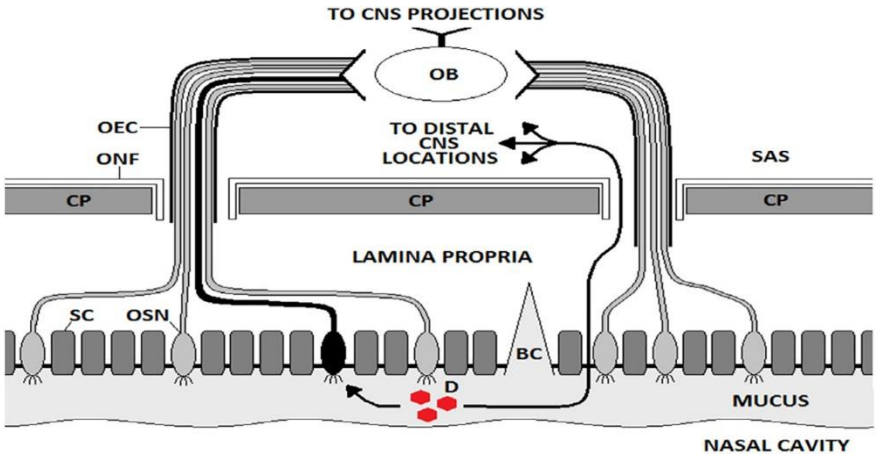
Effective treatments for psychiatric and neurodegenerative diseases have faced many challenges due to the inability of therapeutics to cross the blood brain barrier (BBB). The BBB is made up of endothelial cells and the microvasculature of cerebral tissue essential in protecting against the crossing of neurotoxic substances and maintenance of homeostasis within the central nervous system (CNS). The BBB restricts access to the CNS limiting what can be transported into the CNS to small molecules such as lipophilic compounds or to those that have expressed transporters such as glucose, leptin or ghrelin [130]. The intranasal route of delivery to the CNS uses both an extracellular and intracellular pathway to avoid systemic circulation (Fig. 8). This delivery route engages the olfactory receptor neurons (ORNs), trigeminal nerves, nasal and respiratory vasculature for direct delivery to the brain [131, 132]. Drugs delivered intranasally interact with the respiratory and olfactory areas [133] of the nasal cavity. Both areas enable the transport of compounds directly to the brain via the olfactory neurons. The absorption of molecules occurs at the olfactory and respiratory epithelia [134]. The routes of compound transfer through the olfactory area, of the nares, to the olfactory bulb are transcellular through either the sustentacular cells or the exposed olfactory sensory neurons [134, 135]. The route of transfer to the trigeminal nerves goes through the nasal respiratory epithelium to the brain. The drug is transported to the midbrain from the olfactory bulb or to the brain stem from the trigeminal nerve which may occur via extracellular convective bulk flow [134] or through perivascular routes [136]. Dosed nanoparticles given by intranasal administration have been found in the olfactory bulb 5- minutes after dosing [137] suggesting that the olfactory bulb (OB) is the route of entry for nanoparticle delivery systems.

The Sabban laboratory has demonstrated that intranasal NPY administration to rats reaches the CNS and periphery 30 minutes after administration (Table 2) [81]. The

delivery of NPY to specific brain areas was shown after infusion of fluorescent-labeled NPY (FAM-NPY, Phoenix Pharmaceuticals). Various brain regions, including olfactory bulbs, hypothalamus, hippocampus, locus coeruleus and the amygdala were labeled with FAM-NPY 30 minutes after infusion (Figure 9).

Advantages of intranasal delivery include the ease of administration, noninvasiveness, rapid onset of action and avoidance of effects on cardiovascular function, gastrointestinal degradation and hepatic and extrahepatic degradation (first-pass metabolism).

Figure 8 - Intranasal Pathway Schematic



Adapted from Crowe TP et al., Life Sciences 2018 [138].

Figure 8. Support cells (SC), olfactory sensory neurons (OSN), mucus-secreting Bowman's capsule (BC), cribriform plate (CP), olfactory ensheathing cells (OEC), olfactory nerve fibroblasts (ONF), subarachnoid space (SAS), and olfactory bulb (OB).

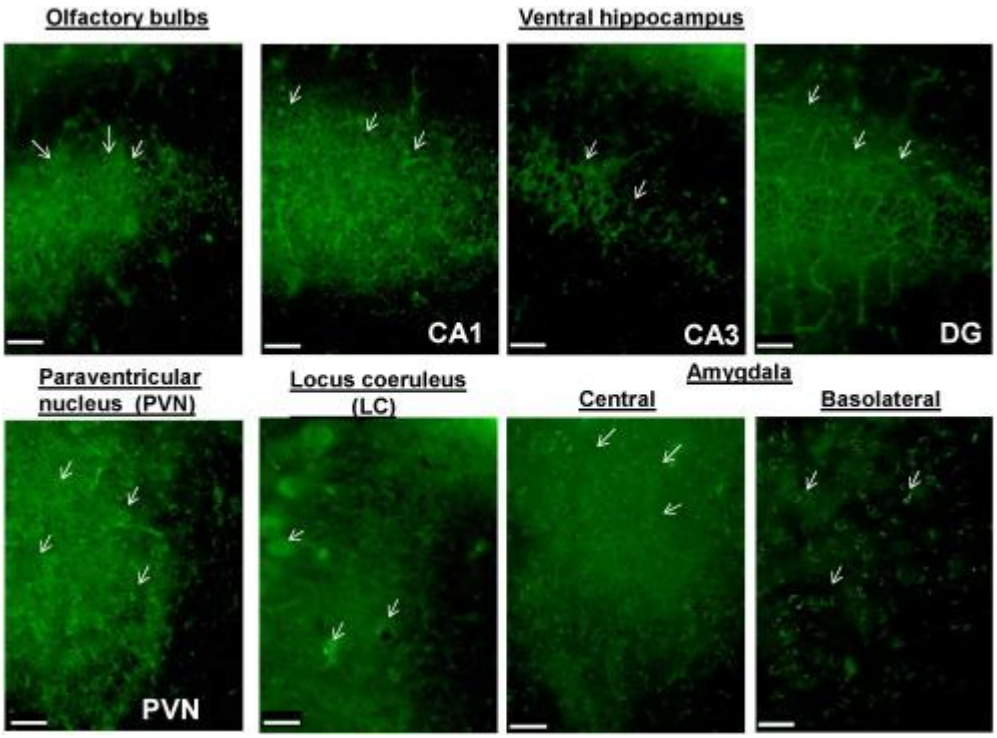
Table 2. Concentration of NPY in CSF and plasma 30 min after IN NPY administration

Treatment	CSF	Plasma
Water	Nd	191 ± 30 ng/ml
NPY, 50 µg	0.4 ± 0.1 µg/ml	211 ± 4.4 ng/ml
NPY, 90 µg	2.6 ± 0.27 µg/ml	206 ± 6.2 ng/ml

Nd = Not detected

Adapted from Serova et al., 2013 [81].

Figure 9 - FAM-labeled NPY Levels in Brain Regions within 30 min after Intranasal Infusion



Adapted from Sabban et al., Neuropeptides 2016 [80].

Figure 9. Fluorescence in various brain regions 30 min after intranasal infusion of FAM-labeled NPY. White arrows show selective labeled cells. Bar = 100 μm.

Animal Models of Stress

Neuropsychiatric disorders such as PTSD, major depression, autism, schizophrenia, major depression and bipolar disorder are very prevalent neurological conditions [139], examples include: anxiety disorders 28.8%; mood disorders, 20.8%; impulse-control disorders, 24.8%; substance use disorders, 14.6% and any disorder, 46.4%. Half of all lifetime cases start by age 14 years and three fourths by age 24 years [139]. These disorders have significant negative effects in society, which is coupled with limited understanding of mechanisms and minimal development of effective therapeutics. The challenge has been in part due to the gap in knowledge in the pathophysiology of these disorders. Although there has been good progress made towards the development of noninvasive technologies to study human brain structure and function, a major challenge has been the ability to study the molecular mechanisms underlying these disorders in the human brain. This limitation necessitates the use of good animal models to recapitulate the disease as seen in humans that can have a good predictive power for drug efficacy in human disease.

Requirements for disease animal models include using causative agents of human disease to exhibit a significant degree of neural or behavioral pathology that corresponds convincingly to the human disease [140]. Several animal models of neuropsychiatric disorders for autism, PTSD, major depression and schizophrenia. have been generated by various methods such as genetic engineering, brain lesions, environmental manipulations and ontogenetic manipulations of relevant brain circuits [141].

To determine whether an animal model is relevant to the specific disorders, it needs to recapitulate the disease or core symptoms as observed in patients. Three types of validators for a disease animal model include: construct validity, face validity, and

predictive validity and has been used widely across many laboratories. In brief, construct validity means that it measures what it intends to measure. The ability to recapitulate important anatomical, biochemical, neuropathological, or behavioral features of a human disease is known as face validity. However, there is no guarantee that an animal model of a neuropsychiatric disorder that would recapitulate all of the behavioral features observed in humans. Animal models cannot mimic psychiatric and somatic disorders in their entirety however; assessing the impact of certain risk factors for the disorders in animal models will facilitate the understanding of their etiology and treatment options in patients.

Response to treatments that can predict the effects of treatments under investigation in humans is known as predictive validity. Behavioral screens using these animal models as a readout for disease symptoms to find drugs for therapeutic intervention have been widely adopted. In our study, we used an animal model of stress, the single prolonged stress animal model (SPS) to recapitulate some of the PTSD core symptoms as seen in patients.

Stress leads to a plethora of many reactions that engage our physiological systems. Various parameters can be utilized for monitoring stress response in animal stress models, these include stress phenotypes and changes that occur during or after each stressor and these should be well characterized.

Chronic or repeated stress is the leading cause of many psychiatric disorders such as major depressive disorder, anxiety disorders and cardiovascular diseases. Although therapeutic agents have been developed to treat stress-related disorders however, they have not been sufficiently effective as many patients often report persistence of core symptoms and side effects. This has put a higher demand on basic

research to search for underlying mechanisms of stress related disorders and effective treatments. Several animal stress models have been developed to study stress related diseases in humans and the development of new drugs.

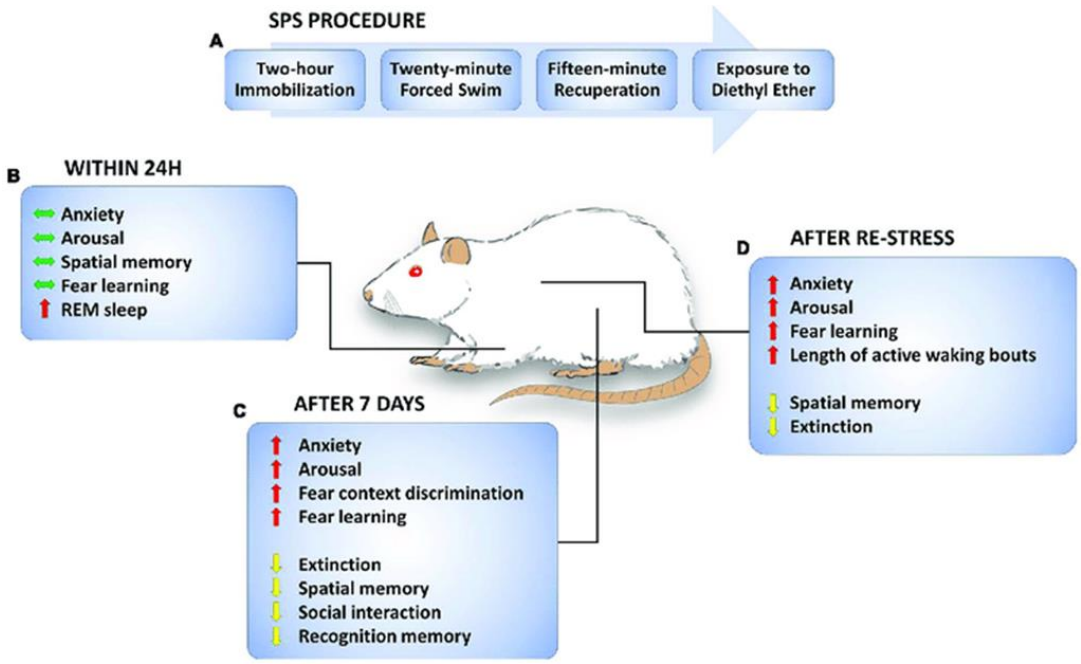
Single prolonged stress (SPS)

Single prolonged stress (SPS) is a multimodal traumatic stress exposure protocol [142]. SPS consists two hours of restraint, twenty minutes forced swim, followed by exposure to ether until loss of consciousness followed by a 7 or 14-day undisturbed period. This protocol is carried out in a single session to cause a robust stress response and elicits a negative feedback of the HPA axis seven days after SPS stressors. Animals exposed to SPS (Figure 10) have many PTSD-like symptoms as seen in patients. This method is reproducible and produces behavioral and neuroendocrine effects similar to those observed in animals exposed to comparable stress protocol [143]. The core symptoms of the SPS model and the time course of PTSD-like progression in these rodents is similar to the impaired neuroendocrine response in PTSD patients [14, 94].

The SPS animal model has been used widely to study behavioral changes, molecular adaptations and neurobiological processes and thus meets the construct and face validity requirement. As a requirement, animal models of post-traumatic stress should recapitulate the disorder and elicit behavioral changes similar to those observed in PTSD patients [144]. The SPS animal model uses a single episode of traumatic stress to evoke persistent behavioral changes comparable to the PTSD core symptoms of anxiety, depressive-like behavior and hyperarousal which are all responsive to PTSD treatments up to three weeks after exposure to SPS [78, 81, 82]. The ability of the SPS animal model to respond to treatments indicates that it meets the predictive validity requirement. Therefore, based on the

numerous and robust studies done using the SPS model and its reproducible nature, we chose this model to study PTSD in rats.

Figure 10 - SPS Schematic



Adapted from Souza et al., Front. Pharmacol. 2017 [145].

Figure 10. Single prolonged stress (SPS) procedure and SPS-induced behavioral changes. (A) Timeline of SPS procedure. On a single day, rats are subjected to a 2-h immobilization followed immediately by a 20-min forced swim. Rats are given a brief period of recuperation and then subjected to diethyl ether until they are anesthetized and unresponsive. (B) Behavioral changes observed up to 1 day later. Anxiety, arousal, spatial memory, and fear learning are unchanged. Acute increase in REM sleep and transition to REM sleep is observed. (C) Behavioral changes 1 week later. Anxiety, arousal, fear context discrimination, and fear learning are increased. On the other hand, extinction, spatial memory, social interaction, and recognition memory are decreased a week after SPS. (D) Behavioral changes following re-stress. Enhanced anxiety, arousal, fear learning, and sleep disturbances remain observed following re-stress, while extinction and spatial memory are impaired. Green, red, and yellow arrows indicate no “changes observed”, “increase”, and “decrease”, respectively.

Methods

Animals

Male Sprague–Dawley rats from Charles River (Wilmington, MA, USA) were used in all experiments. They were housed on a 12 h light/dark cycle at 23 °C with unlimited food and water. Animals (150–160 g, 4 per cage) were allowed to acclimatize for 7 or 14 days and were assigned randomly to experimental or control groups.

All experiments complied with ARRIVE guidelines and were performed in accordance with the NIH 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.

Single prolonged stress (SPS)

Animals were exposed to modified SPS stressors [142] between 9 AM and 2 PM as previously described (Serova et al., 2013). They were immobilized for 2 h by tapping limbs to a metal board, which also restricts motion of the head. This was followed immediately by a forced swim for 20 min in a plexiglass cylinder (50 cm height, 24 cm diameter, Stoelting, Wood Dale, IL, USA) filled two-thirds with 24 °C fresh water. After the forced swim, animals were dried and allowed to recuperate under heat lamp for 15 min and immediately exposed to ether vapor until loss of consciousness. Afterwards, animals were housed two per cage and left undisturbed for 7 or 14 days.

Intranasal infusion

Rats were given intranasal infusion of 150µg and 300µg NPY or 150 µg [D-His^{26}]NPY or 150µg NPY (3–36) or 150µg [Leu31Pro34]NPY freshly dissolved in 20µl distilled water or the vehicle alone under light isolfluorane anesthesia or ether if at the end

of the SPS stressors. Each rat received 10µl into each nostril, and the head kept titled backwards for an additional 15s.

Elevated plus maze (EPM)

Anxiety behavior was tested on the elevated plus maze (EPM) apparatus (Stoelting, Wood Dale, IL) as previously described (Serova et al., 2013) and videotaped. Analyses of all behavioral measurements were performed in a room with dim light setting and animal were allowed to accommodate for 30 minutes in the room. The maze is 40 cm above the floor, has cross-shaped platforms with two open arms with 2 cm high walls, and two closed arms with 40 cm high opaque walls. Rats were placed on the central platform facing an open arm and allowed to explore the maze for 5 min following 30 min acclimation to a room with dim light. Behaviors were recorded and analyzed using tracking software "Viewer 3.0" with a designated "Plug-in" program (Biobserve, Bonn, Germany).

Entry into the open (OA) or closed (CA) arm was defined as entering with all four paws. 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]. Risk assessment was evaluated by the rat poking its head or trunk into an OA while its hind quarters were located in one of the CA. Animals were put in the EPM by an individual blinded to the treatment group. Scoring were done by more than one person that was blinded to the treatment groups. Percent maximal anxiety of one was calculated by X (max anxiety) = # of rats with index 1 x 100% / # of all rats.

Forced swim test (FST)

Immobility time in the forced swim test (FST) was carried out as previously described (Serova et al., 2013) to assess depressive/despair-like behavior. After 30 min accommodation, rats were placed into a plexiglass cylinder (50 cm high, 24 cm diameter)

filled two-thirds with 24 °C fresh water for 5 min and the behavior videotaped. The water was changed between animals. Time spent immobile was defined as the animal showing no movement of any of the four limbs, or only floating movements needed to keep its head above the water for 5 minutes of swim duration.

Social interaction (SI)

The SI test was performed in a room with dim light. One day prior to SI test, animals were allowed to explore an open field (75 cm × 75 cm × 35 cm) for 10 min in order to reduce anxiety component of the novel environment. On the next day, rats were acclimated to the room for 30 min and then allowed to explore the field for 5 min before and 5 min after the introduction of a naïve juvenile rat. The behavior was videotaped and the number of approaches calculated.

Acoustic Startle Response (ASR)

To assess hyperarousal symptoms, ASR was measured in a sound-proof chamber (SR-LAB) (San Diego Instruments, San Diego, CA, USA) as previously described [79, 81]. 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 100 and 115 dB trials for 40ms (total 20 trials) in random order with inter-trial intervals from 30 to 38 s. Voltage data were transferred to a computer using an automated software package (San Diego Instruments). The cylindrical enclosures were cleaned with soap and

water between animals. To analyze data, ASR of animals after single prolonged stress (SPS) were compared to their basal ASR.

Tissue Isolation

Animal were euthanized by decapitation. Brains were dissected used a brain matrix. Sections containing the LC were dissected according to the coordinates 9.2 −10.4 mm posterior to bregma and placed immediately in cold PBS. LC was identified by using reference structures such as the 4th ventricle. A glass pipet with a < 1mm diameter was used to punch out the LC and transferred to an eppendorf tube, which was flash frozen in liquid nitrogen and stored at -80^{o}C until used. The mediobasal hypothalamus, without arcuate nucleus were also isolated immediately following euthanasia of animals. All experimental group of animals including the unstressed controls were treated the same and the surgeon was blind to the experimental groups.

Determination of mRNA levels in the mediobasal hypothalamus and the LC

The RNeasy Mini Kit (Qiagen, Valencia, ML) was used to isolate total RNA and concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific, Pittsburgh, PA). RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) using an oligo dT primer was used in the reverse transcription of 600 ng of LC RNA and 1000 ng of mediobasal hypothalamus RNA. 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; (NET, PPRO6785A-200); (NPY, PPR44428A); (Y1R, PPR56359A), (Y2R, PPR06816A); (Y5R, PPR449006A); (Crh, PPR44803B); GR (NR3c2, PPR52805B); (Fkbp5, PPR51629B); (Crhr1, PPR44886F);and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) forward and reverse priers respectively: 5’ TGGACCACCCAGCCCAGCAAG 3’; 5′-

GGCCCCTCCTGTTGTTATGGGGT-3′ to a final volume 25 µL. Reactions were run on a real-time PCR instrument (Applied Biosystems, Carlsbad, CA) and data were analyzed using QuantStudio™ Design & Analysis Software v. 1.4.1. Data were expressed as the relative fold changes calculated using the ΔΔCt method after normalizing to reference gene GAPDH. The main criterion for a gene to qualify as a reference gene is a stable expression across various cell and experimental settings. GAPDH is a widely used internal control in PCR [146, 147]. GAPDH can also be used alongside other internal controls such as tubulin and beta-actin to assess if GAPDH expression is differentially expressed between similar samples.

Western Blot Analysis

Total protein from selected brain regions was isolated by homogenization in RIPA buffer. Protein concentration was determined by DC Protein Assay (Bio-Rad) with Bio-Tek plate reader. Briefly, 10 µg of total protein were separated on 4%–15% Tris-HCl gradient precast gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). After blocking in Tris-buffered saline (TBS) containing 5% dry milk and 0.1% Tween 20 (TBS with Tween 20 [TBST]), membranes were incubated with primary anti-NET (Abcam 41559) overnight at 4°C. After incubation with secondary anti-Rabbit (IRDye 800CW) signal was visualized using the Odyssey Infrared Imaging System (Li-Cor) and analyzed using IPLab software (BD Biosciences). NET protein levels were normalized to GAPDH. Anti-GAPDH (14C10) was obtained from Cell signaling (Catalogue no. 2118).

Statistical analysis

Data were analyzed using GraphPad (Prizm8, La Jolla, CA, USA) by t-test or one-way analysis of variance (Anova) followed by Tukey's Multiple Comparison Test. The

distributions were analyzed by the Anderson-Darling test for normality and non-parametric Kruskal-Wallis test. When the distribution failed the test of normality and indicated two subgroups, the values were divided based on the mean of the unstressed control before further analysis. Outliers were removed when they were greater than two standard deviations away from the mean. Outliers can arise from low quality of tissue samples or a compromised or sick animal. Values at $p \leq 0.05$ were considered significant.

DNA methylation studies

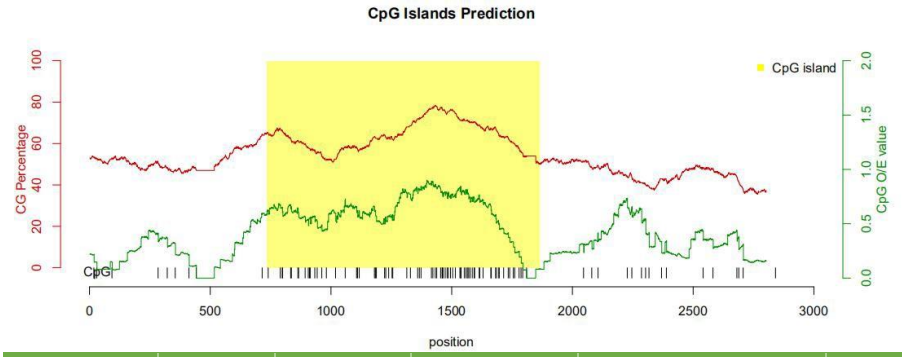
DNA Isolation

Genomic DNA was isolated from the LC using the DNeasy kit from Qiagen. Tissues were kept rocking at 55°C in 500 µL of tissue lysis buffer (100 mM Tris at pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) with 100 µg/mL proteinase K overnight. Digestion was followed by vortexing and centrifugation according to the step-by-step protocol from the DNeasy kit from Qiagen. DNA was recovered and re-suspended in 18 µL of dH2O. To determine DNA quality, eluted DNA was run on 0.9% agarose gel.

Genome Assembly: rn6

Gene	chr	start	end	gene	strand
Slc6a2	chr19	15391682	15431274	Slc6a2	-

We chose the 2000 bp sequence upstream the TSS and 1000 bp downstream the TTS, and predicted the CpG Island. Our prediction is shown in the below figure and table (the red curve is the GC content, the green curve is the O/E value, and the yellow box represents the CpG Island).



Slc6a2					
No.	STAR	LAST	Length	GC_Contents	obs/exp_CpG_ratio
1	735	1863	1129	64.48	0.66

CpG regions sequenced relative to promoter start site are region 1(slc6a2-1) -1199 t0 -947 and region 2 (slc6a2-2) -339 to -614

NET Promoter Sequence:

>Slc6a2:chr19:15430274-15433274:+

CCCTTCAGAAAGGAGCCCGAGTCTACCTCCGTGGTGCTGTATGTGGCCT

GTGGGTTCATTCCAGAGCAGATGGGTAAAATGACAGCTCATCACGAAGA

GGACAAAAGG

GCTTGTCTGCTTGGTGTCTTCTCCAGGCTTGTATGCACTGGGCTTCACAA

CTTGGCCAGAGCTTGCAAAGCTAACAAGGACACTCCAGTCCCCAGGAGT

GCCTCAAGG

TCCCATTCAGCTCTTGGCATCCAGTATCCTGATCTCATGAAAGCTGTGTA

CACTTACCCCTGTAGCCCGCTGAGGATCCAAGGGCTCTCATTTTATCCAG

CCTAACGC

CTGAGCTCCAAGGTTGAGTGCTAGAATTATCGTCAAATCTTGTGTCTTGA

CTGGAACATCCCAAACTTTTCTCTACTCCCTTTTCTGCGTCACAGCATTG

ACTCTGAG

TCCCTGGCCCGACCAGATGCCTTCTATGCTGTCCCTGAGCCCTTGTGCCT

ACTACTATTCAAGGTTCTGCCTCTATTTCCCTAACAGAAACTACCCAGAT

CCTTTTTC

TCAGTACCCTTAAGAGTTCTTTCCTGCACACAAAATTTTTTCTGTGTCTCC

TCTTTTCCTCCATGTGCATCCCTGCTCCCTCCCCAGACAATGCCACAAGA

ACAGGCA

GCATCCCAAAAGCTTCAGCCAACCCTGTGCTCACCACCACCATTCTTGTA

GCAGAGATAGGGGAAGCGCCACACATTGGCCAGGTCCACTGCGAAGCC

CACCACAGAC

AGCAGGAAATCAATCTTCTTGCCCCAAGTCTCCCGGGGCTGCGCGTCAC

CATCCTGGGATGCCAGGAGACACTGGACGCCGTTGTGCTCCTTCACCAC

TAGCAGATCG

GCGGTTTTGCAGGGCCTCAGGGGCTGCTCGGGCAGCTGGTCCGCCCCGC

CGAGCTCAGGCTGCACCTGCGGCTTCATTCGTGCCAGAAGCATGGGTGC

GGCTGGAGAG

AGGGACTTCGGAGGCACTGGGGACACACCAAAGTTCAAGTTCTGCTCGT

ATGAAAGGAGAAAAAGCAGTCAACAAGGCTTGCCTCCCGTTTGCACCAA

CTGAGACCAC

CCAAGGGAACCCAGATGTCAGGATCCGTGCGCTGAGCGGACTCAGGCTT

CCTAGAAAAGGAGTGCCTGAGTTGGGAATTTTTGGACACTAAGGTTGTG

ACGTGCGTCC

GACTGGGACAGAATTTATAGGTGGGGACCTCCTCGGGCCGCCCTGTGGG

TCGGGCTAAAGACCCCGCCGAGGTCTACCTGGGTCATTCACTGTCCCCTC

CCCTTCCCT

AAATGCCAAGTTTCTTGCGGGCCCTGAGGGACCGAAGTTGACTGGAGGC

CCCTATCCAGCTCCGGGGAACGCCCTCCGACAGCACTAGGAGCCTTTGG

GGACACTCTA

GAGGGTGCAGCCGCTCACGATGTGCCTCGGAGCCGGTCTGCACACTTAC

CGGTCCCGCGCGCACAGAGCGCACTCACGTGGCGGACAGCACGGGGAT

CCCGGGCTCCA

GCCGGGGCTGGGGCTGGGCTCCGCCGCGTGGCACTCAGGCGGGGCCCGG

GAGCGCTGCGGGGACGGGGTGGCCGGCAGGACGCGGGCTGGCTGGCTC

TGGCCGCGCCA

GGAGGAGGCCGACCTGGTAATGTAGCCTGTGCCCCAGGTAACGCGCCTA
TTGCATTAGCCCCGCGCCCAGCCCGCTCGGTCTGGAGCCTGTTAGCGCTA
ATGCAGACT
GACAGCGTGGCCGGGACTGGCAGAGGCGGGGGCGCAGCTCCCAAGGGC
TCCGGGGTGGCGGGTGCCGGACTCTCAGAGACTCGGGGGATCTGGCTTA
GCACCCTTTCC
AGGCTGCTCCCCAAAACACCTGGCTCTGACCTCTGGACAGAGGAAGAGA
GCAACAGATTCCCAGAACTCCCAGAAGTCTGTCAAAGGGGAGTTGTAAA
GACACAGGGA
AGCAGTCACCACCCCAGTGACACCAGCATTGTCATACACCCAAGCCACT
TAACCTACTTGGTGAGAGTTTTCTCCACCAGCAGAATGGGAACTTAGTG
CTCCGAGATG
AAGACTGTGACTGGTGGCCTGCAGGCTCGGCCTTTTGGGTAGTTTTGTTT
GGCCGGCTCTGCTGGTTGAAAGGTCATAGACAGATTTTACTCCTGGCTCC
ATTTGTGA
CCAGGCTGTGACCTTGATCTTGGGGTCTGTGGCCCTGATCTATAAGGCTG
AGAACTGATACCCACTCGTTCTCAAAAATGCTCCCCGTATGCCAAATCA
AGCCTTTTT
AGTTTCCCACTAGTGGACGACACCTTCAAAGAGCCGGTTGTTTGGTCCCG
GAAACAGGAGGGAAACAGGAAACACAATAAATAAATACTTATTACTCA
CTCGTGTGGT
ATGGACTGGGGGCGGGTAAAGCAAAGTAAGAAAATGGATGGAAAAAGC
AAAGTAGGGCTGCACATTTTTAACAATATGTTCTATAGTGTTTAAGCATG
AACTACTTG

ACCTGAGAGTTTACTTACCCAACTTCTTGTGTCTGAATCAACCCTGAAAGG
AAGATCGCAGAGACAATCCCAGACACCCAGTGTGCAAAGAGCTTACCGCC
CTTAGAA

CTGCTTGTGGTCATAGGAGAACAGGACTTTGGGGAAGCCAAATTAGTAAG

GTCAGCAGGCAGGAATGTAGGGAAGACACAAAACAGTACGGGAAGACGC

TTAGATAAG

CCTAGCCGCACTGATCTTTCTACACTTTCAACATTCCAAGCTCAAAGCTAC

CCATGGGCACTTGCATTAGCTCTGACTTCCATCTAAGTTGTTCAGGTCTTA

GTGAAG

ATATTCCTTCTTAGACTTGTGCATCTAGAAGTCGCCCCCATCCTCCATCAG

ATCACTGATGCAATTTATTAATTATTAATTAATTAATAATTGATTAATTGAT

TAATAATTGATCTCCC

TTTTAGTGGCAGTTGAGCTCTAGAAGAACCAAGTATTGTCTACCTTATCCA

CCTGTGTCTCTGGCACTAGAACTCACTAAACACT

DNA Library Preparation was performed by CD Genomics, (Shirley, New York).

In brief, gene-specific DNA methylation was assessed by a next generation sequencing-based bisulfite sequencing PCRs (BSP). In brief, BSP primers were designed using the online MethPrimer software. Genomic DNA (1 ug) was converted using the ZYMO EZ DNA. Methylation-Gold Kit (ZYMO) and one-twentieth of the elution products were used as templates for PCR amplification. For each sample, BSP products of multiple genes were generated, pooled equally and subjected to adaptor ligation. Barcoded libraries from all samples were sequenced on the Illumina Hiseq platform using paired-end 150 bp strategy.

Primer Sequence for two CpG regions in the NET promoter

Amplicon Name	Primer Pair	Length	GC %	Tm
Slc6a2-1	GTTATTATTTTGGGATGTTAGGAGATATTGGA	32	0.31	59.5
	CAAACCTTATTAACTACTTTTTCTCCTTTCATAC	34	0.29	59.7
Slc6a2-2	AGTTTTTGGGGATATTTTAGAGGGTGTAG	29	0.38	60.6
	TTACCTAAAACACAAACTACATTACCAAATC	31	0.29	58.1

Amplicon Sequence Information

Slc6a2-1 (Region 1)

5’GTCACCATCCTGGGATGCCAGGAGACACTGGACGCCGTTGTGCTCCTTCAC
CACTAGCAGATCGGCGGTTTTGCAGGGCCTCAGGGGCTGCTCGGGCAGCTGG
TCCGCCCCGCCGAGCTCAGGCTGCACCTGCGGCTTCATTCGTGCCAGAAGCA
TGGGTGCGGCTGGAGAGAGGGACTTCGGAGGCACTGGGGACACACCAAAGT
TCAAGTTCTGCTCGTATGAAAGGAGAAAAAGCAGTCAACAAGGCTTG---3’

Slc6a2-2 (Region 2)

5’AGCCTTTGGGGACACTCTAGAGGGTGCAGCCGCTCACGATGTGCCTCGGAG
CCGGTCTGCACACTTACCGGTCCCGCGCGCACAGAGCGCACTCACGTGGCGG
ACAGCACGGGGATCCCGGGCTCCAGCCGGGGCTGGGGCTGGGCTCCGCCGC
GTGGCACTCAGGCGGGGCCCGGGAGCGCTGCGGGGACGGGGTGGCCGGCAG
GACGCGGGCTGGCTGGCTCTGGCCGCGCCAGGAGGAGGCCGACCTGGTAAT
GTAGCCTGTGCCCCAGGTAA---3’

Next generation sequencing-based bisulfite sequencing PCR (BSP)

Gene-specific DNA methylation was assessed by a next generation sequencing-based BSP, according to previously published method [148]. In brief, BSP primers were designed using the online MethPrimer software. 1 μg of genomic DNA was converted using the ZYMO EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) and one-twentieth

of the elution products were used as templates for PCR amplification with 35 cycles using KAPA 2G Robust HotStart PCR Kit (Kapa Biosystems, Wilmington, MA, USA). For each sample, BSP products of multiple genes were pooled equally, 5'-phosphorylated, 3'-dA-

tailed and ligated to barcoded adapter using T4 DNA ligase (NEB). Barcoded libraries from all samples were sequenced on Illumina platform.

For the bisulfite sequencing reads of each sample, firstly, adapters and low-quality reads were removed using software Trimmomatic-0.36 software [149] essentially has a variety of processing steps that removes low quality sequences such as adapter sequences, primer fragments that could result in suboptimal downstream analyses. After removing the adapter sequences and filtering out the low quality reads, the clean sequencing reads were directly aligned to the target sequences using software Bsmap (v2.73) with the default parameters, which combines genome hashing and bitwise masking to achieve fast and accurate bisulfite mapping. Methylation levels are defined as the fraction of read counts of 'C' in the total read counts of both 'C' and 'T' for each covered C site. On the basis of such read fraction, methylated cytosine was called using a binomial distribution as in the method described by Lister et al. [148] whereby a probability mass function is calculated for each methylation context (CpG, CHG, CHH). Two-tailed Fisher's Exact Test was used to identify cytosines that are differentially methylated between two samples or groups. Only those CG covered by at least 200 reads in at least one sample were considered for testing. P-value thresholds were selected such that the level of Significance is less than 0.001.

Statistical analysis

The methylation level of the CG on each target sequence of each sample was calculated. The methylation level is calculated as follows: methylation rate of C site = 100* supports for

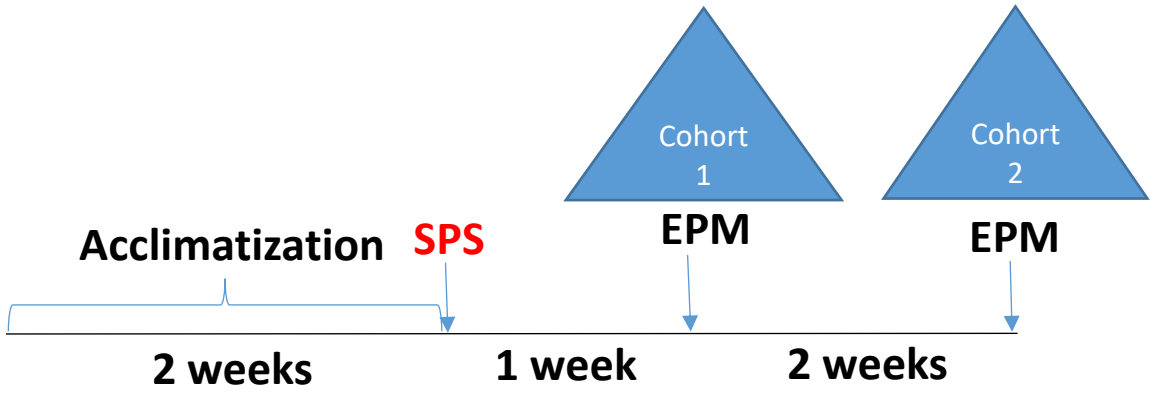
methylation reads/ (supports for methylation reads + support for unmethylated reads). The level of methylation in each sample at each site was calculated, the significance of the differences between the sample groups was based on the methylation level of each

site. The average level for each CpG locus were calculated from (methyl_C/(methyl_C+N_methyl_C), the methyl_C means number of reads that have methylated CpG while the N_methyl_C means the number of reads that have non methylated CpG. Data were analyzed using GraphPad (Prizm8, La Jolla, CA, USA) by t-test.

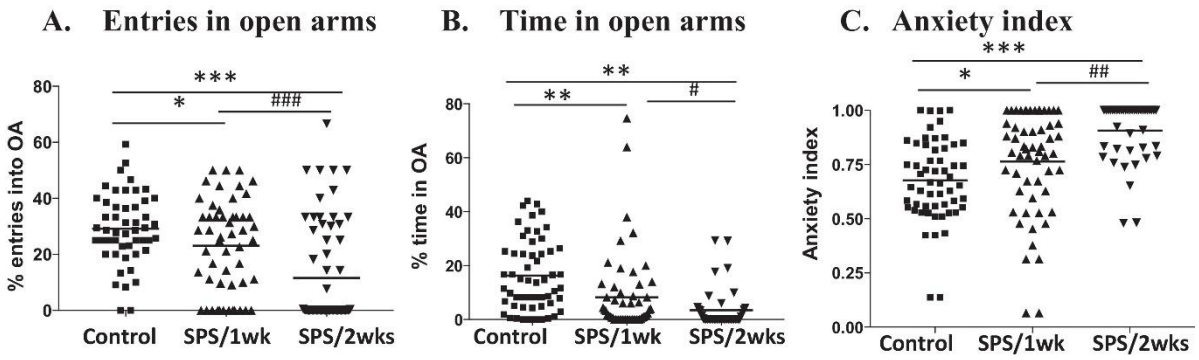
Results

Measure of anxiety on the elevated plus maze (EPM) one or two weeks after SPS stressors

Anxiety behavior is a core PTSD symptom that can last for a lifetime. Anxiety symptoms have delayed onset and might not manifest immediately after the trauma. It has been shown that evaluating the biological progression of PTSD and comorbid impairments using a staging approach is necessary to understand the underlying mechanisms of the disease [5]. To evaluate the progression of anxiety symptoms in the SPS animal model of PTSD, two cohorts of animals were placed in the EPM and behavior was assessed one and two weeks after SPS.



Progression of anxiety-like behavior



D. Percentage of rats with maximal anxiety Index (1.0)

Groups	Number of rats	Percentage
Controls	n = 56	3.6
SPS/1 week	n = 57	17.5
SPS/2 weeks	n = 42	57.1

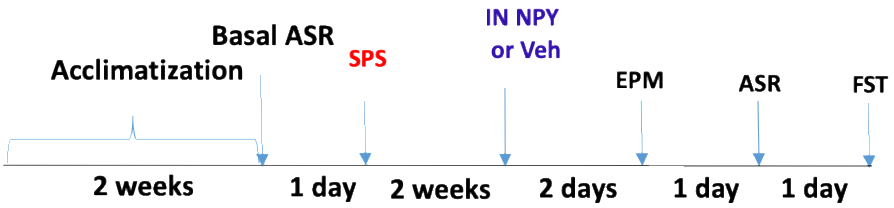
Figure 11. Anxiety index at one or two weeks after SPS. A. Percentage of entries into the open arms (OA) of the EPM. B. Percentage of time in the OA of the EPM. C. Anxiety index; each data point represents an individual rat with mean (horizontal line). *p < 0.05, **p < 0.01, ***p <0.001 compared to Controls; #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. Each point represents values for individual animal.

There was a worsening of anxiety-like behavior at two weeks (11A-C). ANOVA showed significance in the percent of entries into the OA ($F = 18$; $p < 0.05$), duration in the OA ($F = 14$; $p < 0.001$) and the anxiety index ($F = 6$; $p < 0.001$). Tukey comparison of the means revealed that all SPS groups had fewer entries and lower duration in the OA and higher anxiety index compared to controls ($p<0.001$) in Figures 11A and B. The SPS groups after SPS stressors differed in the OA entries and duration ($p < 0.001$; $p <0.05$) and anxiety index ($p < 0.01$) at one week compared to two weeks. This result revealed a progressive worsening of anxiety like behavior in the SPS animal model indicated by the increase in the number of animals with maximal anxiety index of 1 at two weeks compared to one week in Figure 11C.

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

Previously Dr. Sabban's laboratory showed that intranasal NPY prevented development of PTSD symptoms and also reversed symptoms when intranasal NPY was administered one week after SPS. Here, we wanted to evaluate the ability of intranasal NPY to reverse SPS-triggered impairments in anxiety and depressive-like symptoms manifested two weeks after SPS stressors when symptoms are more severe (Fig. 11).

Animals 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 when symptoms are more severe, subsequently we doubled intranasal NPY dose to 300 µg /rat.



Reversal of anxiety-like behavior by NPY

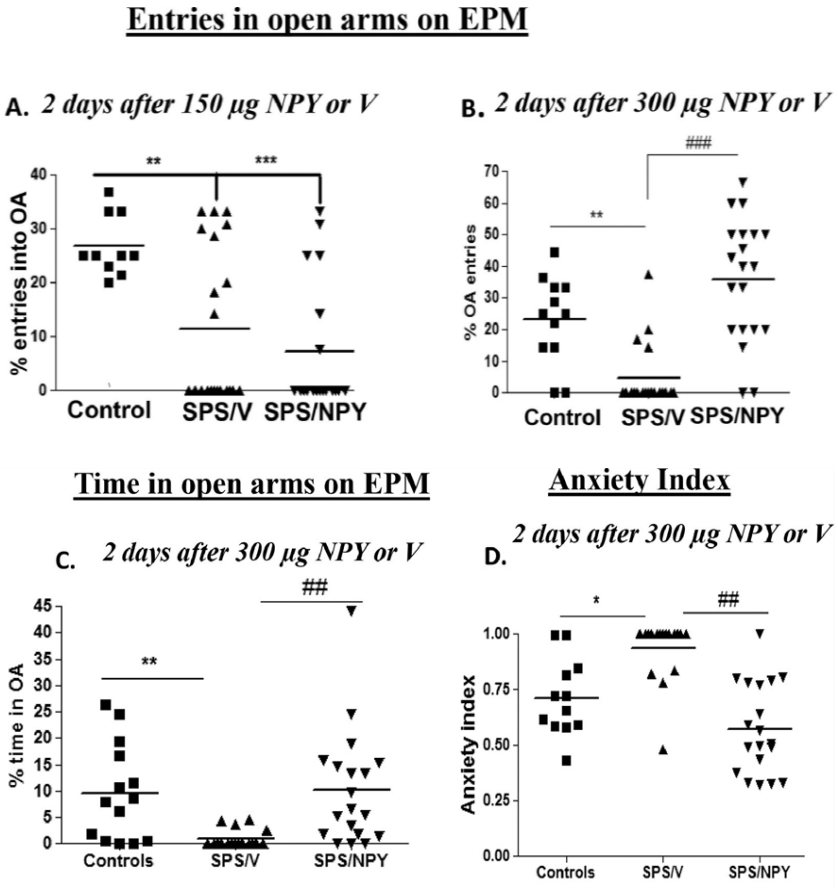


Figure 12. Effect of intranasal administration of NPY to reverse symptoms of anxiety on the EPM. A. 150 µg/rat of NPY (SPS/NPY) or vehicle (SPS/V) two weeks after SPS stressors compared to unstressed controls (Controls). B–D Rats were administered 300 µg/rat of intranasal NPY or vehicle 2 weeks after SPS stressors and tested on EPM for: B. entries into open arms; C. time in open arms and D. anxiety index. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to Control; $^{\#}p <$

0.05, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for SPS/V vs SPS/NPY. Each point represents values for individual animal.

Intranasal NPY dose of 150 µg /rat was previously shown to be effective in reducing SPS-elicited anxiety behavior one week after SPS stressors (Serova et al., 2014a) however, intranasal administration of 150 µg /rat of NPY was not sufficient to reverse anxiety symptoms manifested two weeks after SPS stressors (Figure 12A) . One-way ANOVA ($F = 7.2$ $p < 0.003$) was significant for percent of entries into the OA. There was no difference in percent entries to the open arms in rats given vehicle or NPY, and both groups had less entries to the OA compared to the unstressed controls.

The dose of intranasal NPY was doubled to 300 µg /rat in a different cohort (B-D) and anxiety-like behavior was assessed on the EPM two days later. One-way ANOVA revealed significant effect of treatment on entries into OA ($F = 20.5$, $p < 0.0001$, B); time spent in OA ($F = 6.7$ $p < 0.01$, C) and anxiety index ($F = 19$, $p < 0.001$, D). The NPY-treated rats behaved similarly to the unstressed controls in the number of entries to the OA, time spent in the OA and anxiety index.

Figure 13

Effects of NPY on Risk Assessment and Locomotion

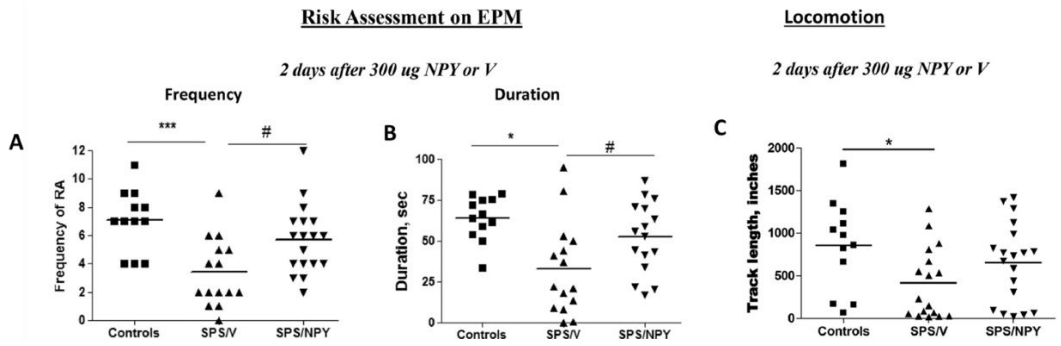


Figure 13. Effects of intranasal administration of NPY on risk assessment: number of head dips and locomotion: total distance travelled on the EPM. In A-C, rats were administered 300 µg/rat of intranasal NPY or vehicle 2 weeks after SPS stressors and tested on EPM. A. Frequency; B. duration of risk assessment on the EPM and C. 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.

Infusion of 300 µg/rat of intranasal NPY treatment also increased frequency ($F = 8.4$, $p<0.001$, Fig. 13A) and duration ($F = 6.7$, $p<0.05$, Figure 13B) of risk assessment to levels similar to controls ($p < 0.05$, Figures 13A and 13B). There was a significant difference in locomotor activity as measured by track length on the EPM ($F = 3,2$, $p<0.05$, Figure 13C). Unstressed controls traveled longer distance than the SPS vehicle group as expected. Administration of intranasal NPY did not have a significant effect on the locomotion.

Figure 14

1.2.3 Reversal of Depressive-like behavior in the Forced Swim Test by NPY

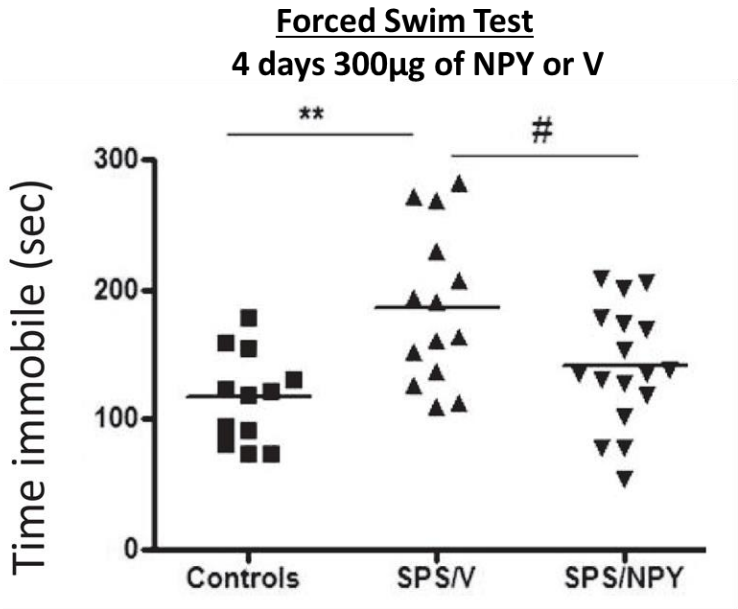


Figure 14. Reversal of depressive-like symptoms. A. 300 µg/rat of intranasal NPY were administered to rats (SPS/NPY) two weeks after SPS or vehicle (SPS/V), two days afterwards they were tested on EPM. Rats were then subjected to the forced swim test (FST) two days after EPM. Each data point represents an individual rat. **$p < 0.01$; Control; #$p < 0.05$.

As shown in Figure 14, 300 µg/rat of intranasal NPY had a protective effect against depressive-like behavior by reducing the immobility time in the FST. One-way ANOVA ($F = 7.1$, $p<0.01$) was significant for the FST. The SPS/V group spent more time immobile compared to the unstressed controls. Tukey multiple comparisons of the means revealed significant differences between the SPS/V group and controls ($p <0.01$) or the SPS/NPY ($p<0.01$) group.

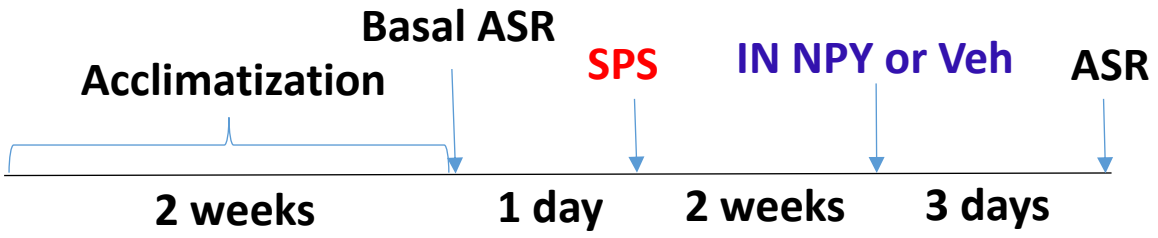
Results presented here demonstrate a time-dependent progression in the development of anxiety symptoms in the SPS model of PTSD. Severe anxiety, as indicated by limited duration and entries into open arms of EPM and high anxiety index, was much more pronounced at two weeks, compared to one week following the traumatic stress, suggesting a worsening of symptoms. Doubling of the dose that was previously shown to be effective one week following SPS stressors (150 µg /rat) was necessary to reverse anxiety and immobility on the FST two weeks after SPS.

Reversal of Hyperarousal Symptoms in Acoustic Startle Response by NPY

Startle response consists of a brief reflex twitch of somatic muscles in response to a sudden, intense stimulus such as a sharp noise, that is dependent upon brainstem cochlear nuclei, the caudal pontine nucleus, and spinal motor neurons [150-152]. Poor extinction and abnormal acoustic startle response (ASR) has been shown to be associated with PTSD, thus potential predictors of the development of PTSD-like behavior. Here, we evaluated if intranasal administration of NPY can reverse hyperarousal symptoms and how long such reversal can be sustained.

Figure 15

Acoustic startle response (ASR)



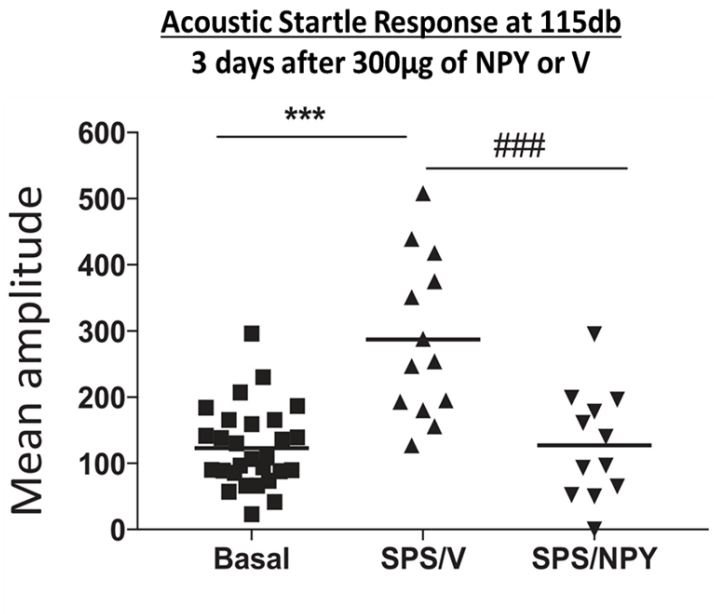


Figure 15.

Reversal of hyperarousal symptoms. A. 300 µg/rat of intranasal NPY were administered to rats (SPS/NPY) two weeks after SPS or vehicle (SPS/V), three days afterwards ASR was measured. Each data point represents an individual rat. ***p < 0.001; Control; ###p < 0.001.

Administration of intranasal NPY reversed hyperarousal symptoms. Two weeks after exposure to SPS, rats were administered either intranasal 300 µg NPY/rat (SPS/NPY)

or vehicle (SPS/V) and three days later ASR was measured. One-way ANOVA revealed significance in ASR ($F = 18.6$, $p<0.001$). The SPS group that received vehicle treatment had an elevated startle response ($p<0.001$), while those given intranasal NPY had ASR levels similar to the basal pre-SPS levels and lower than those only given vehicle ($p< 0.001$).

Figure 16

1.2.5 Sustained Reversal of Hyperarousal Symptoms in Acoustic Startle Response by NPY.

Figure 8

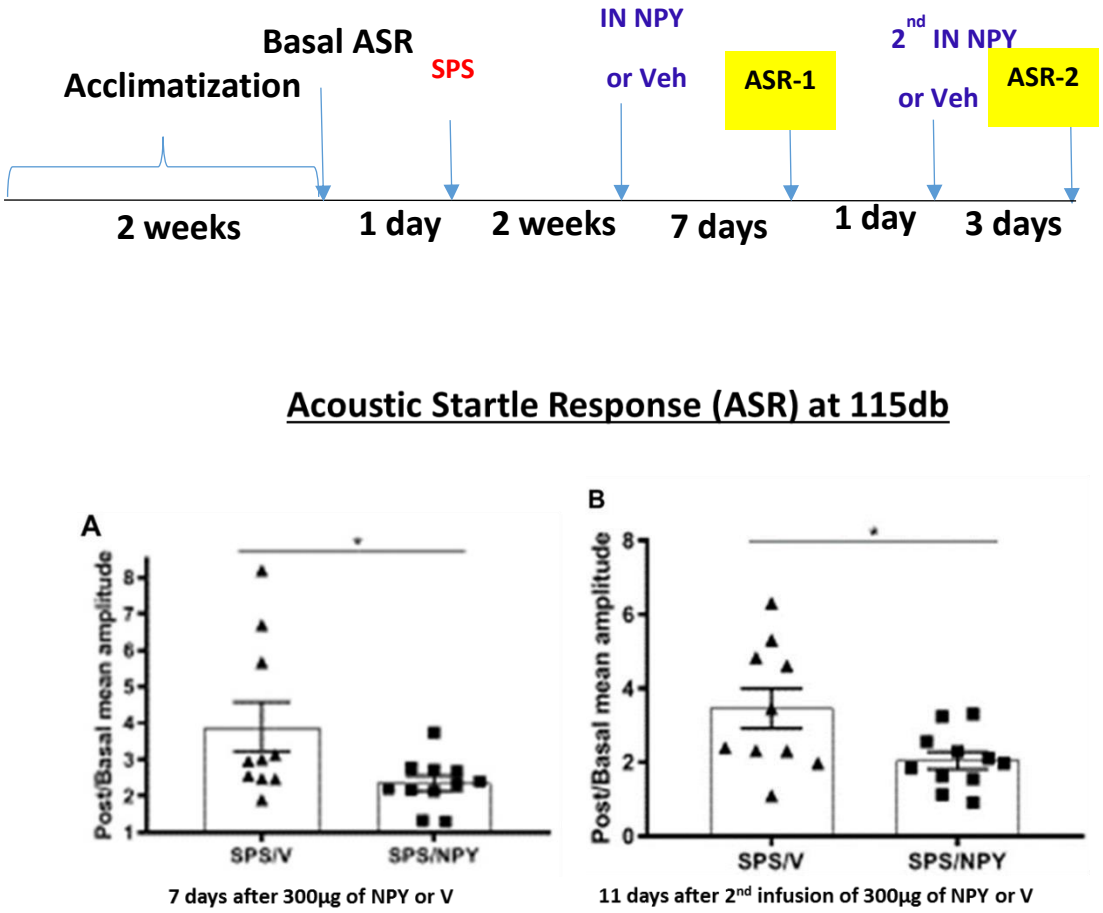


Figure 16. Sustained reversal of ASR by NPY. (A) Two weeks after SPS stressors, rats were given intranasal administration of 300 µg NPY or vehicle and ASR measured 7 days after NPY infusion. ASR after SPS was normalized to the basal ASR for each animal and the ratio (ASR/Basal ASR) is plotted. (B) A second infusion was given after the first infusion in the same cohort and ASR measured 3 days later (eleven days after the first NPY administration). ASR after second infusion was normalized to the basal ASR for each

animal and the ratio (ASR/Basal ASR) is plotted. Each point represents values for individual animal. $^{*}P < 0.05$, $^{**}P < 0.01$.

Intranasal NPY sustained the reversal of the SPS-triggered rise in ASR. NPY reduced ASR seven days after its first administration (16A) shown by a lower startle response ($t_{(19)} = 2.3$, $P < 0.05$) compared to the vehicle treated group (SPS/V). In addition, we assessed the effect of a second infusion of NPY (16B) and found that NPY indeed sustained the decrease in acoustic startle response 11 days after the first infusion ($t_{(22)} = 2.1$, $P < 0.05$).

In summary, the ASR results demonstrates that doubling intranasal NPY was sufficient to reverse the SPS-elicited rise in startle response and hyperarousal symptoms and maintained basal arousal levels for more than 1 week..

Effect of Selective NPY receptor agonists on development of SPS triggered impairments

Experiment design for agonists

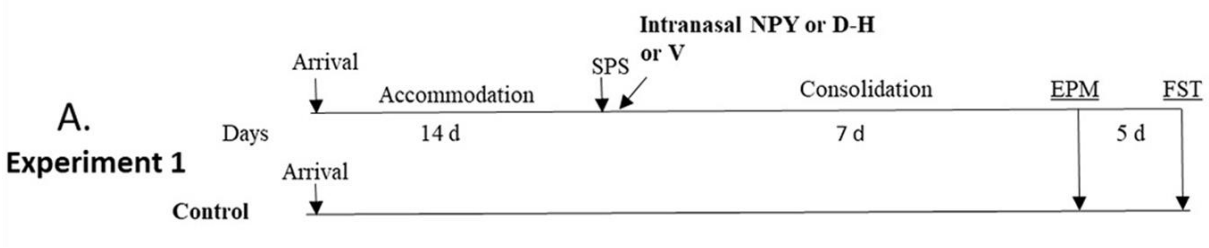
The neuropeptide Y (NPY) mediate its effects via several G-protein coupled receptors to proffer resilience to the harmful effect of stress in post-traumatic stress disorder (PTSD). To investigate the role of individual NPY receptors in the resilience effects of NPY to traumatic stress, intranasal infusion of either Y1R agonists [D-His^{26}]NPY, [Leu^{31}Pro^{34}]NPY, Y2R agonist NPY (3-36) or NPY was administered to male Sprague-Dawley rats immediately following the last stressor of the single prolonged stress (SPS) protocol. We assessed effects one-week post SPS because we have determined that at this time point PTSD symptoms in the SPS animal model are not as severe. When efficacy is observed for a particular agonists one week after SPS, we would test the effectiveness of the agonist two weeks after SPS when symptoms have become more severe.

Dosing concentrations: NPY generally has a high affinity to Y1R, Y2R and Y5R. The affinities of [D-His^{26}]NPY, [Leu^{31}Pro^{34}]NPY, and Y2R agonist NPY (3-36) for Y1R, both Y1R and Y5R and Y2R respectively are similar to NPY affinities (Table 6). We began by using a similar dose that have been proven effective with NPY. Future experiments will utilize a dose dependent approach in consideration of receptor affinities to further determine the most efficient doses for optimal effectiveness.

One-week post SPS

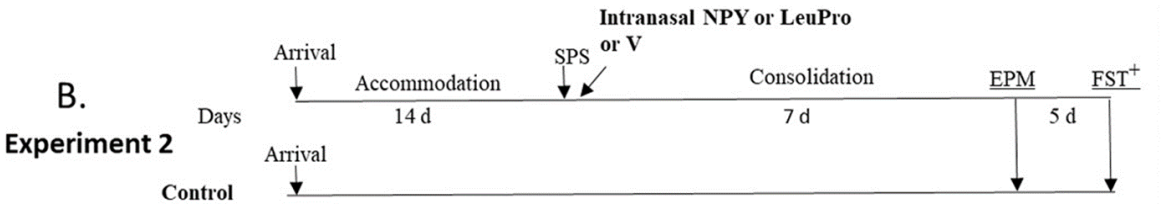
Experiment 1. The scheme of experiment is shown in Figure 17A. Rats (n = 12 per group) were infused intranasally with either [D-His^{26}]NPY (150 μg/rat) (D-H) or NPY (150 μg/rat) or vehicle immediately following the last stressor (ether) of the SPS protocol. Animals were left undisturbed for 7 days and behavior on EPM and FST were assessed afterwards.

Figure 17A



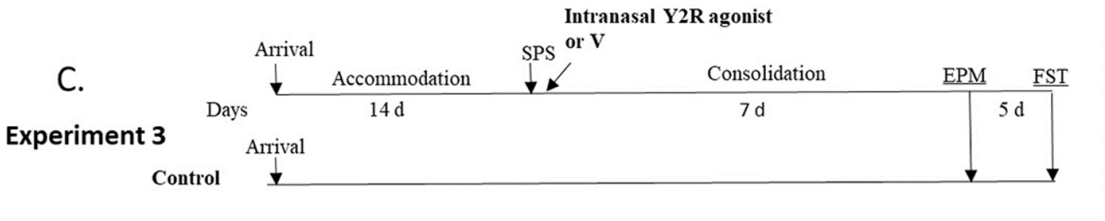
Experiment 2. The scheme of experiment is shown in Figure 17B. Rats (n = 10 rats per group) were infused intranasally with either [Leu^{31}Pro^{34}]NPY (150 μg/rat) (LeuPro) or NPY (150 μg/rat) or vehicle following the last stressor (diethyl ether) of the SPS protocol. Animals were left undisturbed for 7 days and behavior on EPM and FST were assessed afterwards.

Figure 17B



Experiment 3. The scheme of experiment is shown in Figure 17C. Rats (n = 12 rats per group) were infused intranasally with either the Y2R agonist, NPY(3–36) (150 μg/rat) or vehicle immediately following the last stressor (diethyl ether) of the SPS protocol. Animals were left undisturbed for 7 days and behavior on EPM and FST were tested afterwards.

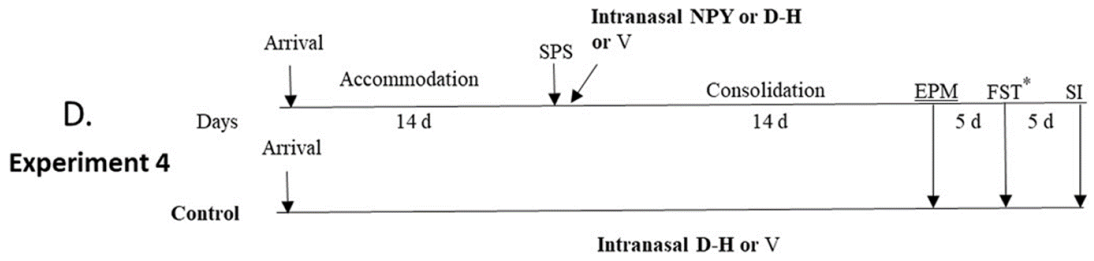
Figure 17C



Two weeks Post-SPS

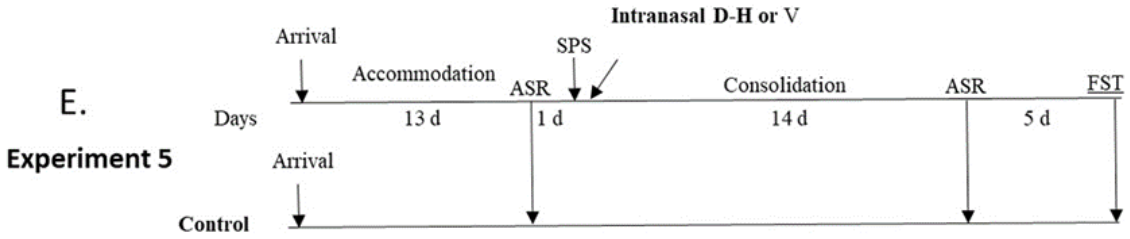
Experiment 4. The scheme of experiment is shown in Figure 17D. The rats (n = 12 rats per group) were infused intranasally with either NPY (150 μg/rat), or [D-His^{26}]NPY (150 μg/rat) or vehicle immediately following the last stressor (diethyl ether) of the SPS protocol. Animals were left undisturbed for 14 days and behavior on EPM, FST and social interaction were assessed afterwards.

Figure 17D



Experiment 5. The scheme of experiment is shown in Figure 17E. Rats (n = 12 rats per group) were infused intranasally with either [D-His^{26}]NPY (150 μg/rat) or vehicle immediately following the last stressor (ether) of the SPS protocol. Animals were left undisturbed for 14 days and behavior on acoustic startle response (ASR) and FST were tested afterwards.

Figure 17E



Result from Experiment 1 (schematic is shown in Figure 17A).

Effect of Intranasal administration of NPY Y1R agonist [D-His^{26}]NPY on hyperarousal and depressive-like behavior.

We examined the ability of [D-His^{26}]NPY, in comparison to NPY, as an early intervention to prevent the development of anxiety and depressive like behavior one week after rats were subjected to SPS stressors (Figure 18A-D). Intranasal administration of either [D-His^{26}]NPY (150 μg/rat) or NPY (150 μg/rat) or vehicle were administered immediately following the last stressor (ether) of the SPS protocol. As shown in Figure 18A, the anxiety index differed significantly among the groups ($F(3, 41) = 14.54$, $p < 0.0001$). Tukey multiple comparison test indicated that the vehicle treated group (SPS/V) had a higher anxiety index compared to the unstressed control group ($p < 0.001$), the NPY treated group (SPS/NPY) ($p < 0.001$) and the [D-His^{26}]NPY treated group (SPS/D-H) ($p < 0.0001$). In Figure 18B, One-way ANOVA was significant ($F(3, 38) = 4.718$, $p < 0.01$) for time spent in the open arms (OA). Tukey multiple comparison test showed that the SPS/D-H group spent more time ($p < 0.05$) in the OA than the SPS/V and the SPS/NPY ($p <0.05$) groups. One-way ANOVA was also significant ($F(3, 41) = 3.135$, $p <0.05$) for the number of entries into the OA. Tukey multiple comparisons showed that the SPS/NPY group had more entries ($p < 0.05$) into the OA compared to the SPS/V group however, neither the SPS/D-H nor unstressed control groups differed from each other or any of the other groups (Figure 18C).

Furthermore, animals were tested for immobility on the FST as shown in Figure 18D. One-way ANOVA revealed significance ($F(3, 38) = 10$, $p < 0.0001$). Tukey multiple comparisons showed that the SPS/V rats were immobile for a longer time ($p < 0.001$) than the unstressed control group or the SPS/D-H group ($p < 0.0001$). The SPS/D-H group spent less time immobile ($p <0.05$) compared to the SPS/NPY group. No significant differences

were observed in the time spent immobile between the NPY treated SPS group and the group given vehicle. In conclusion, NPY Y1R agonist [D-His^{26}]NPY prevented SPS elicited anxiety and depressive-like behavior.

Figure 18

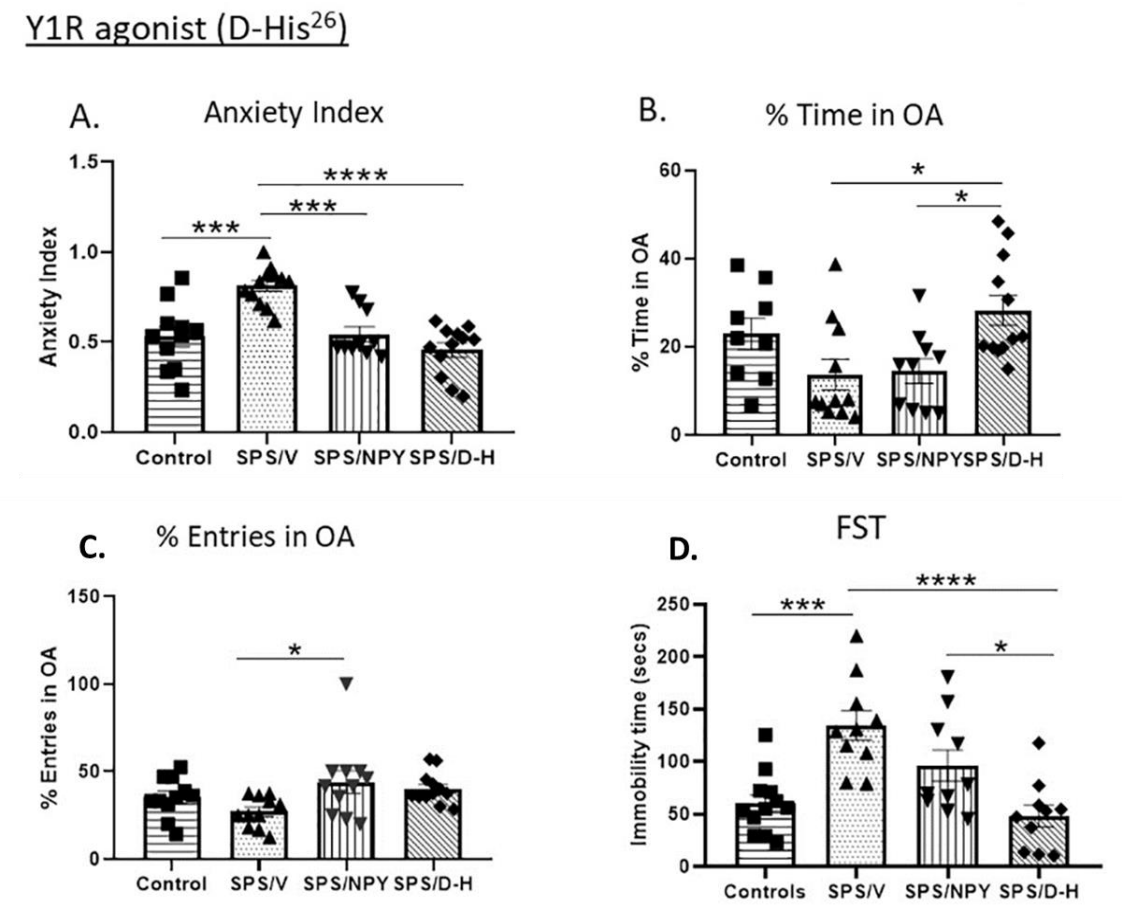


Figure 18. Effects of Y1R agonists on anxiety and depressive-like behavior. Rats were unstressed (Control), or exposed to SPS and immediately after the last stressor (ether) rats received intranasal infusion of 150 μg/rat of NPY (SPS/NPY) or 150 μg/rat of [D-His^{26}]NPY (SPS/D-H). Animals were tested on the EPM and FST for effect of [D-His^{26}]NPY after seven days on: anxiety index (A); time in OA (B); number of entries into the OA (C); Immobility time in the FST (D).

Result from Experiment 2 (schematic is shown in Figure 17B).

Effect of Intranasal administration of [Leu^{31}Pro^{34}]NPY on anxiety behavior

Intranasal infusion of either [Leu^{31}Pro^{34}]NPY (150 μg/rat) or NPY (150 μg/rat) or vehicle was administered immediately following the last stressor (diethyl ether) of the SPS protocol (Figures 19E-G). One-way ANOVA, ($F(3, 32) = 4.241$, $p < 0.05$) revealed significance for anxiety index on the EPM. Tukey multiple comparison test showed that the SPS/V ($p < 0.05$) and the SPS/LeuPro ($p < 0.05$) had a higher anxiety index compared to the unstressed control group (Figure 19E). Results for time spent in the OA (Figure 19F) were significant by one-way ANOVA ($F(3, 31) = 9.165$, $p < 0.001$). However, Tukey multiple comparison tests were not significant for time spent in the OA between the SPS/LeuPro and SPS/V groups. Both the SPS/V and SPS/LeuPro groups spent less time ($p < 0.001$) in the OA compared to the unstressed control group. The SPS/NPY group also spent less time ($P < 0.05$) in the OA compared to the unstressed control group. One-way ANOVA was not significant for the number of entries into the OA (Figure 19G). Intranasal administration of Y1R agonist [Leu^{31}Pro^{34}]NPY did not prevent SPS elicited anxiety. It is also important to note that although NPY had been effective in previously published studies, this batch of NPY was later confirmed to be of a poor quality by the manufacturer and might explain why it wasn't effective in the studies presented here.

Figure 19

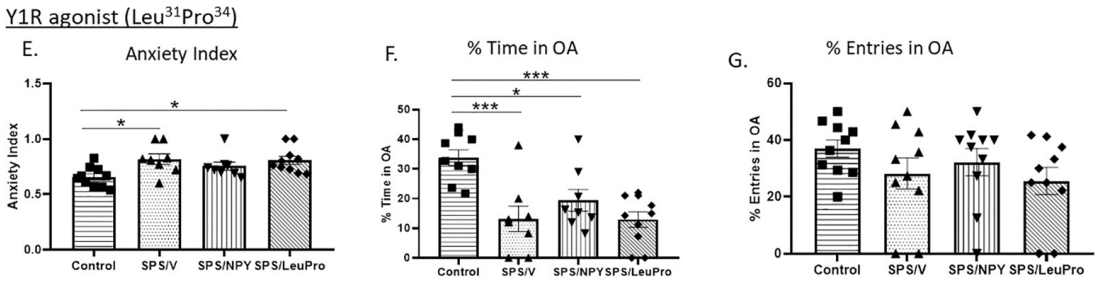


Figure. 19. Effects of Y1R receptor agonist on anxiety behavior. Rats were unstressed (Control), or exposed to SPS and immediately after the last stressor (ether), 150 μg/rat of [Leu^{31}Pro^{34}]NPY was administered intranasally. One week later the following measures were tested on the EPM: anxiety index (E); time in OA (F); number of entries into the OA (G). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Result from Experiment 3 (schematic is shown in Figure 17C).

Effect of Intranasal administration of NPY (3-36) on anxiety and depressive-like behavior

Intranasal infusion of either a Y2R (NPY 3–36) agonist (150 μg/rat) or vehicle was administered immediately following the last stressor (diethyl ether) of the SPS protocol (Figures 20 H-K). One -way ANOVA indicated significance ($F(2, 29) = 8.362$, $p < 0.001$) for anxiety index in Figure 10H. There was no difference in anxiety index between the Y2R agonist treated group (SPS/Y2) and the vehicle treated group (SPS/V) by Tukey multiple comparisons. Both the SPS/Y2 and SPS/V groups had higher anxiety index ($P < 0.05$) than the unstressed control group. In Figure 20I, one-way ANOVA revealed significance ($F(2, 31) = 6.270$, $p < 0.01$) for time spent in the OA. There was no difference between the SPS/Y2 and SPS/V groups for time spent in the OA, both groups spent less time ($p < 0.05$) in the OA compared to the unstressed control group. In Figure 20J, one-way ANOVA was significant ($F(2, 26) = 15.43$, $p < 0.0001$) for number of entries into the OA. There was no difference between the SPS/Y2 and SPS/V groups for number of entries into the OA by Tukey multiple comparison test. Both the SPS/V ($p < 0.001$) and the SPS/Y2 ($p < 0.05$) had fewer number of entries into the OA compared to the unstressed control group. In conclusion SPS elicited anxiety or depressive-like behavior was not prevented by the intranasal administration of Y2R agonist (NPY 3–36).

Figure 20

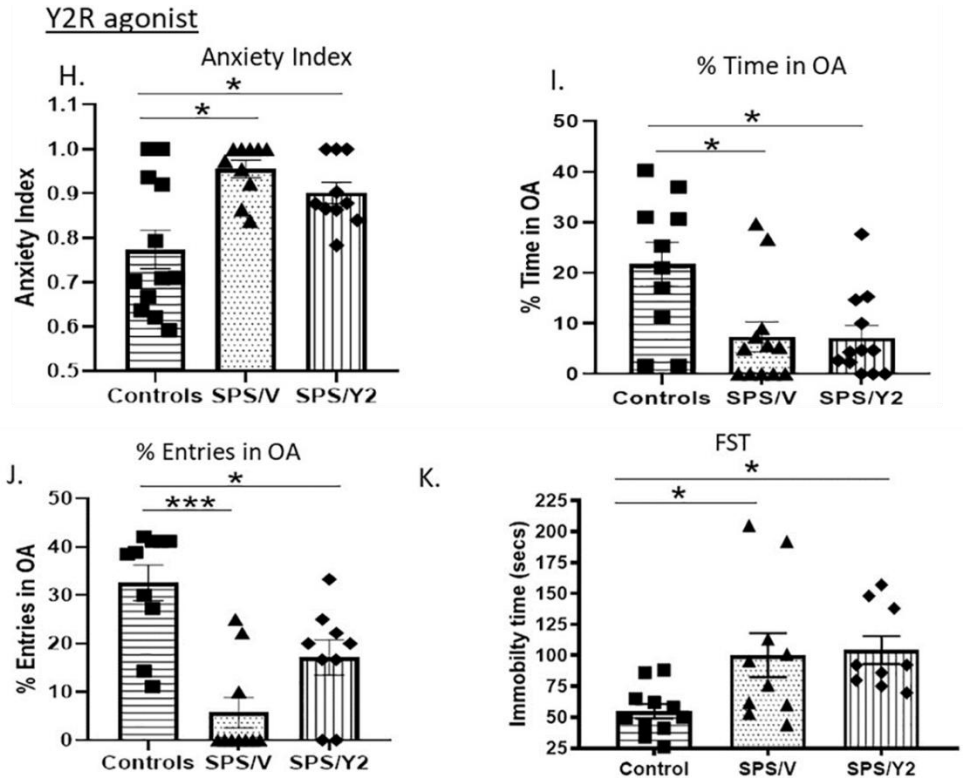


Figure. 20. Effects of Y2R receptor agonist on anxiety and depressive-live behavior. (NPY 3–36) on: anxiety index (H); time in OA (I); number of entries into the OA (J); immobility time in the FST (K). Each point represents values for an individual animal. Means ± SEM are shown. *p < 0.05; **p < 0.01; ***p < 0.001.

Result from Experiment 4 (schematic is shown in Figure 17D).

Effect of Intranasal administration of NPY Y1R agonist [D-His^{26}]NPY on anxiety and social interaction

Previously we showed that the number of animals displaying severe anxiety after SPS increased three-fold from one week to two weeks (Serova et al., 2019), indicating progressive worsening of anxiety symptoms. Here we evaluated if [D-His^{26}]NPY maintained its efficacy as a potential early intervention therapy 14 days after SPS when the anxiety phenotype is more severe (Figures 21A-C). Intranasal infusion of either NPY (150 μg/rat), [D-His^{26}]NPY (150 μg/rat) or vehicle was administered immediately following the last stressor (diethyl ether) of the SPS protocol. One-way ANOVA revealed significance ($F(3, 39) = 3.592$, $p < 0.01$) for anxiety index (Figure 21A). Tukey multiple comparison test showed that only the [D-His^{26}]NPY treated group (SPS/D-H) had a lower anxiety index ($p < 0.05$) compared to the vehicle treated group (SPS/V). One-way ANOVA was also significant ($F (3, 33) = 3.370$, $p < 0.01$) for time spent in the OA. The SPS/D-H group spent more time ($p < 0.05$) in the OA compared to the SPS/V group (Figure 21B) by Tukey multiple comparison test. The number of entries into the OA (Figure 21C) was also significant ($F (3, 43) = 3.617$, $p < 0.01$) by one-way ANOVA. The SPS/D-H group had more number of entries ($p < 0.05$) into the OA compared to the SPS/V group.

In addition, one-way ANOVA was also significant ($F (3, 34) = 4.188$, $p < 0.01$) for social interaction (Figure 21D). Tukey comparisons showed that [D-His^{26}]NPY treatment prevented the impairment in social interaction elicited by SPS in the SPS/D-H group ($p < 0.01$) compared to the vehicle treated group (SPS/V). In conclusion SPS elicited anxiety and social impairment two weeks after SPS was prevented by intranasal administration of NPY Y1R agonist [D-His^{26}]NPY.

Figure 21

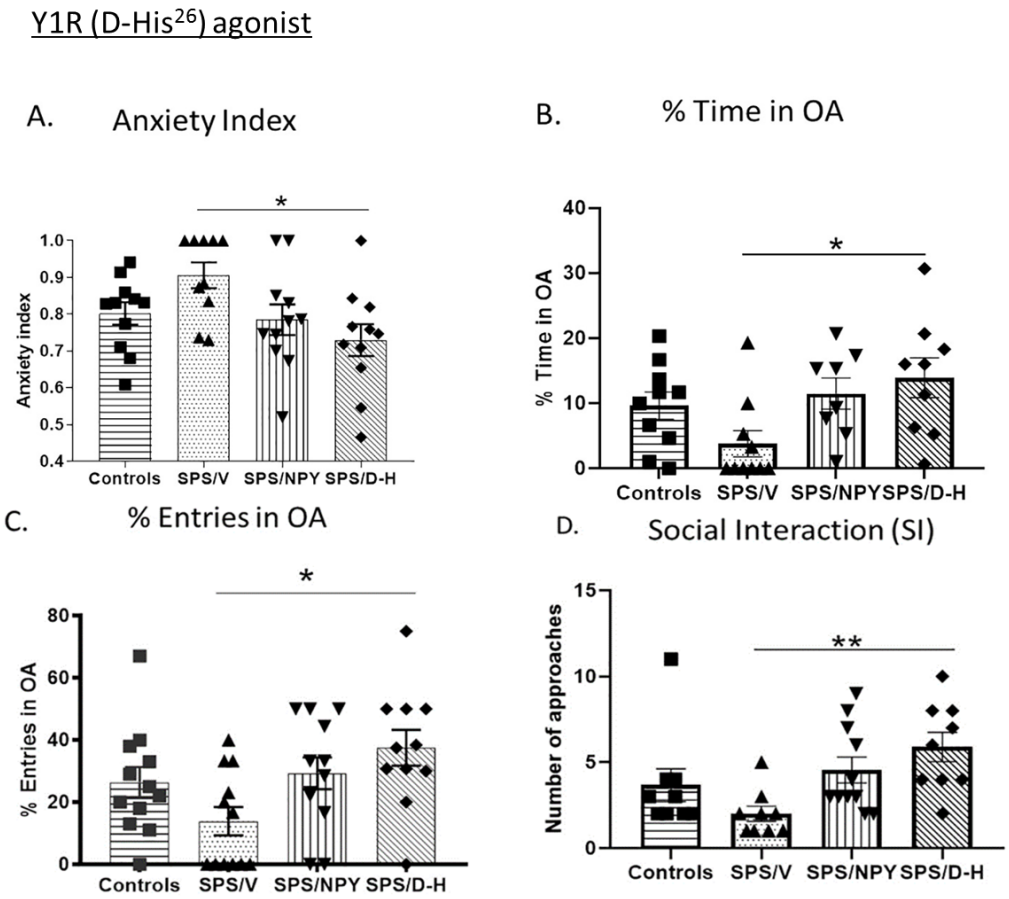


Figure. 21. Effects of [D-His^{26}]NPY and NPY receptor agonists on anxiety behavior. Rats were unstressed (Control), or exposed to SPS and immediately after the last stressor (ether) rats received intranasal infusion of 150 μg/rat of NPY (SPS/NPY) or 150 μg/rat of [D-His^{26}]NPY (SPS/D-H) or vehicle (SPS/V). After 14 days they were tested on the EPM and the effect of [D-His^{26}]NPY on: anxiety index (A); time in OA (B); number of entries into the OA (C) and social interaction (SI) (D). Each point represents values for an individual animal. Means ± SEM are shown. *$p < 0.05$; **$p < 0.01$.

Result from Experiment 5 (schematic is shown in Figure 17E).

Effect of Intranasal administration of NPY Y1R agonist [D-His^{26}]NPY on hyperarousal and depressive-like behavior.

Intranasal infusion of either [D-His^{26}]NPY or vehicle was administered immediately following the last stressor (diethyl ether) of the SPS protocol. Two weeks after SPS they were tested for acoustic startle response (ASR) in Figure 22E and five days later, they were subjected to FST (Figure 22F). One-way ANOVA revealed significance ($F(2, 43) = 3.724$, $p < 0.05$) in the ASR. Tukey multiple comparison test showed increase in the ASR over basal pre-SPS levels for the vehicle treated group (SPS/V) ($p < 0.05$) but not the [D-His^{26}]NPY treated animals (SPS/D-H). One way Anova was significant ($F (2, 27) = 6.226$, $p < 0.01$) in the FST two weeks after SPS. The SPS/V group spent more time immobile than the SPS/D-H group ($p < 0.01$) and the unstressed control group ($p < 0.05$) by Tukey multiple comparison test.

These results show that intranasal administration of Y1R agonist [D-His^{26}]NPY can protect against depressive/despair behavior and impaired social interaction in a rodent PTSD model two weeks after symptoms are more severe.

Figure 22

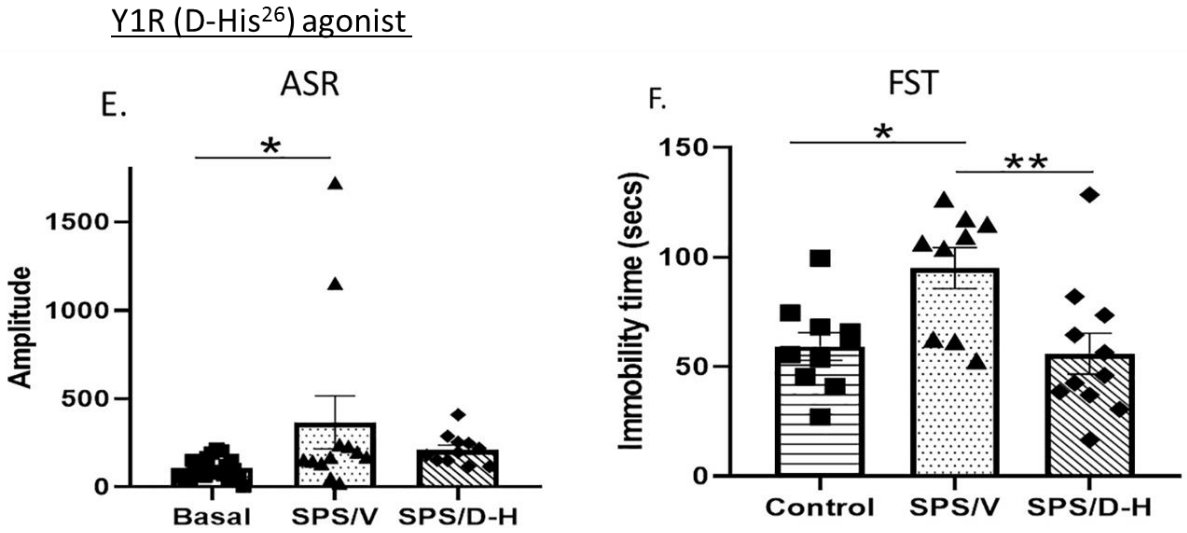


Figure. 22. Effects of [D-His^{26}]NPY receptor agonist on acoustic startle response (ASR) (E); immobility time in the FST (F). Each point represents values for an individual animal. Means ± SEM are shown. *$p < 0.05$; **$p < 0.01$.

In summary these results suggest that Y1R is a key mediator of NPY's protective effects on anxiety and depressive-like symptoms emanating from exposure to severe stress. Activation of the Y1 receptor protected against the development of anxiety, impairments in social interaction and depressive/despair-like behaviors. [D-His^{26}]NPY was especially effective compared to [Leu^{31}Pro^{34}]NPY. In the FST, [D-His^{26}]NPY was more effective than NPY in decreasing immobility time in the FST.

Gene expression changes of CRH and NPY systems in the LC and Mediobasal hypothalamus two weeks after SPS

The HPA axis is a classic neuroendocrine loop. Neurosecretory neurons located in the medial parvocellular paraventricular nucleus (PVN) of the hypothalamus initiates the secretion of glucocorticoids (Antoni, 1986; Whitnall, 1993). Glutamatergic and noradrenergic transmissions innervate the PVN stimulating CRH release to activate the HPA axis. Enhanced innervation is associated with chronic stress [153]. PVN neurons are divided into parvocellular and magnocellular divisions while CRH neurons in the PVN are mostly the parvocellular division (Aguilera, 1998: Sawchenko et al., 1985). GRs are also expressed by most neurons expressing CRH in the rat PVN (Agnati et. al., 1985). Inhibitory effect of GRs on CRH following repeated stress show a correlation between the decrease in GR mRNA levels in the PVN and increased CRH levels (makino et al., 1995). In addition, the LC is also an important target for the PVN CRF projections. PVN neurons projecting to the LC are positive for CRF immnunorecativity (Ryes et al., 2005) and have their synapses on both catecholaminergic and non-catecholaminergic cells in the LC. Ascending LC norepinephrine containing projections innervate multiple sites in the brain, including the PVN, amygdala, hippocampus and the cerebral cortex (Hwang et al., 1998). Stressful events activate the LC leading to an increase of norepinephrine (NE) release in these brain regions, making it possible that the LC can influence different brain functions

and behaviors that include vigilance, attention, arousal memory acquisition, locomotor control and response to stress (Aston-Jones et al., 1996; Berridge and Waterhouse, 2003; Morilak et al., 2005; Sara, 2009; Valentino and Van Bockstaele, 2008).

Therefore, we speculated that PTSD core symptoms observed in the SPS animals could result from the progressive dysregulation of some of these neuronal networks. We evaluated molecular changes in both LC and mediobasal hypothalamus following SPS because these two brain regions have been implicated in mediating responses to stress arising from the dysregulation of the HPA axis. It was previously shown that stress increased tonic LC-NE activity an event necessary and sufficient for stress-induced anxiety and aversion (McCall et al., 2015). The PVN located in the medial basal hypothalamus is required for activation of the HPA axis and is implicated in the development of pathologies arising from the dysregulation of the HPA. Furthermore, the LC is highly responsive to stressful stimuli that activate the HPA axis. Therefore, investigating molecular changes in these brain regions could shed some light on genes that respond to chronic stress in the SPS animal model.

Changes in the CRH and NPY system in the LC

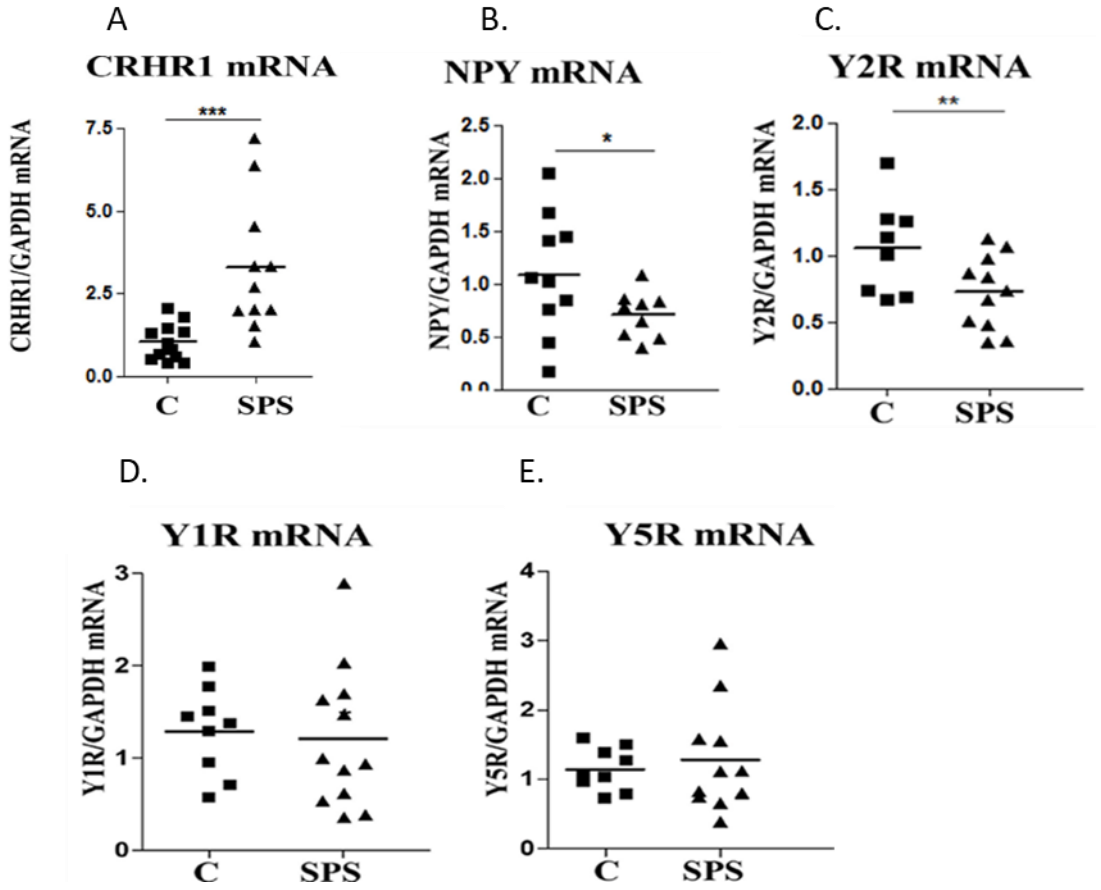


Figure 23. Changes in gene expression in locus coeruleus at two weeks after exposure to SPS stressors. A**.** CRH receptor 1 (CRHR1) mRNA levels. B. NPY mRNA. C. Y2R mRNA. D. Y1R mRNA. E. Y5R mRNA

We examined changes in the gene expression of the CRH and NPY systems two weeks after exposure to SPS stressors in two separate experiments by t-tests. CRHR1 mRNA was elevated in the SPS animals compared to the unstressed controls ($p < 0.001$, Figure 23A) with wide variation among the animals. The SPS group had lower NPY mRNA levels compared to the unstressed controls ($p < 0.05$, Figure 23B). Similarly, there was a reduction in the Y2R mRNA levels in the SPS group two weeks after SPS compared to the unstressed controls ($p < 0.01$, Figure 23C). By contrast, no changes were observed in levels of Y1R and Y5R mRNAs two weeks after SPS stressors (Figures 23D and E).

Changes in the CRH and NPY system in the mediobasal hypothalamus

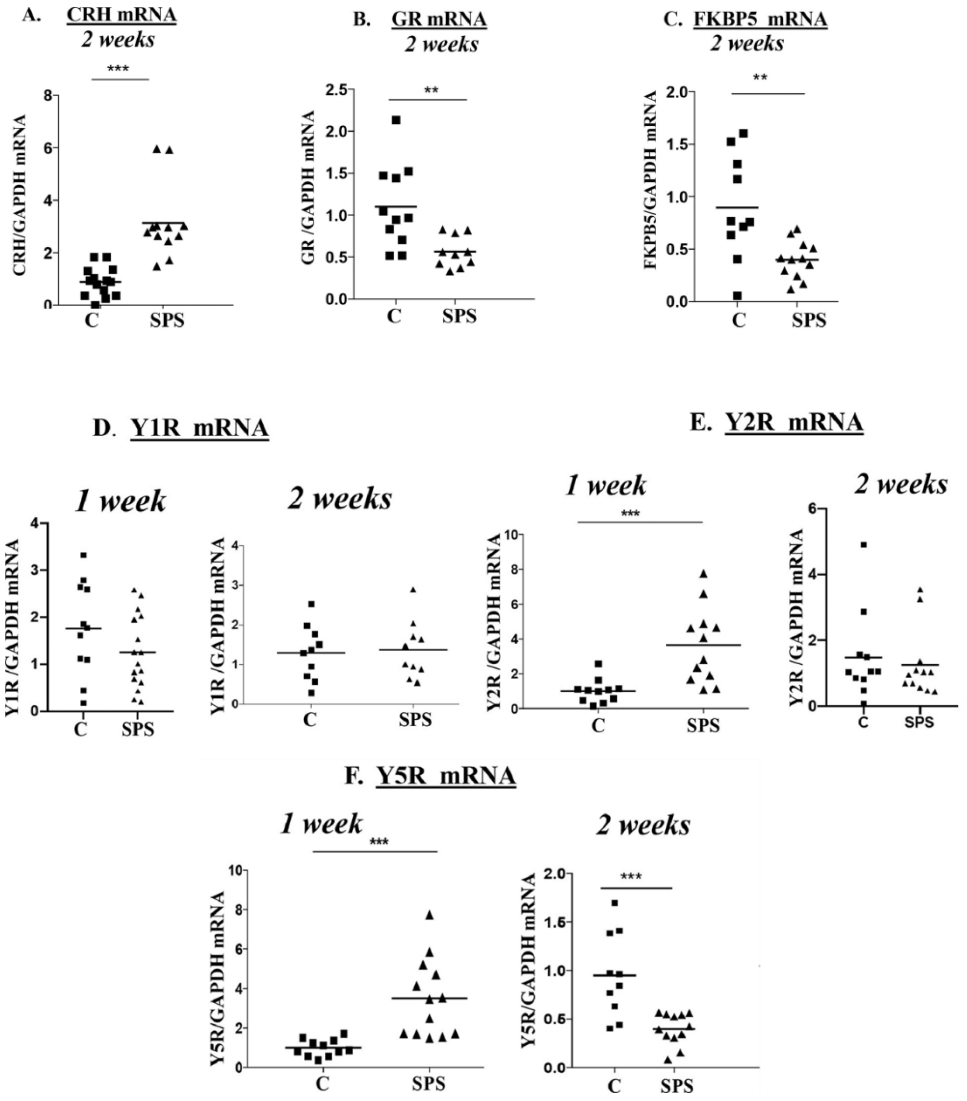


Figure 24. Changes in gene expression of CRH, GR and FKBP5 in the mediobasal hypothalamus after exposure to SPS stressors. A. CRH, B. GR, and C. FKPB5 two weeks after SPS. D. Y1R mRNA. E. Y2R mRNA and F. Y5R mRNA levels at one and two weeks after the SPS. Data points represent an individual rat with mean (horizontal line). **p < 0.01, ***p <0.001.

In the Medio basal hypothalamus CRH mRNA levels were elevated over the unstressed controls (Figure 24A, $p < 0.001$). The mRNA levels for GR (Figure 24B) and FKBP5 (Figure 24C) were decreased ($p < 0.01$) compared to the unstressed controls. The effect of SPS on levels of gene expression of NPY receptors in the mediobasal hypothalamus were evaluated one and two weeks after SPS. Y1R mRNA levels were unchanged (Figure 24D) however, changes in Y2R (Figure 24E) and Y5R (Figure 24F) mRNAs were observed one week after the traumatic stress of SPS. After two weeks, Tukey multiple comparison test revealed that only the Y5R was significantly ($p < 0.01$) reduced in the SPS group compared to the unstressed control (Figure 24F).

Overall, these results show pronounced changes in the CRH and NPY gene expression system in both the LC and mediobasal hypothalamus 2 weeks following a single exposure to SPS stressor (summarized in Tables 3-5).

Table 3. **Changes in mRNA expression in the LC**

2 weeks post SPS

Increase	Decrease	No Change
CRHR1	NPY, Y2R	Y1R, Y5R

Table 4. **Changes in mRNA expression in mediobasal hypothalamus**

I week post SPS

Increase	No Change
Y2R, Y5R	Y1R

Table 5. **Changes in mRNA expression in mediobasal hypothalamus**

2 weeks post SPS

Increased	Decrease	No Change
CRH	GR, FKBP5, Y5R	Y1R, Y5R

Table 6. Representative qPCR program

Block Type	96-Well 0.2-mL Block	
Calibration	Yes	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	No	
Calibration	01-31-2019	
Calibration	Yes	
Calibration	01-31-2019	
Calibration	Yes	
Calibration	01-31-2019	
Chemistry	SYBR_GREEN	
Date Creat	2020-02-21 15:16:57 PM PST	
Experiment	Barcode	
Experiment	Comment	
Experimen	F:\2020-02-21_SPS67_LC_NET.eds	
Experimen	2020-02-21_SPS67_LC_NET	
Experimen	2020-02-21 11:45:36 AM PST	
Experimen	Comparative CT (ΔΔCT)	
Instrument	272521109	
Instrument	272521109	
Instrument	QuantStudio™ 5 System	

Table 7

Representative Amplification Raw Data

Well	Well Posit	Cycle	Target Name	Rn	Delta Rn
1	A1	1	Target 1	0.658	-0.013
1	A1	2	Target 1	0.663	-0.010
1	A1	3	Target 1	0.672	-0.002
1	A1	4	Target 1	0.676	-0.001
1	A1	5	Target 1	0.680	0.001
1	A1	6	Target 1	0.684	0.003
1	A1	7	Target 1	0.685	0.002
1	A1	8	Target 1	0.685	0.000
1	A1	9	Target 1	0.685	-0.002
1	A1	10	Target 1	0.687	-0.002
1	A1	11	Target 1	0.690	-0.001
1	A1	12	Target 1	0.695	0.002
1	A1	13	Target 1	0.704	0.009
1	A1	14	Target 1	0.720	0.023
1	A1	15	Target 1	0.754	0.054
1	A1	16	Target 1	0.819	0.118
1	A1	17	Target 1	0.946	0.242

Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye.

Table 8

Representative Melting Point Raw Data

Well	Well Posit	Reading	Temperature	Fluorescence	Derivative	Target Name
1	A1	1	60.000	1,347,148.125	31,605.090	Target 1
1	A1	2	60.313	1,338,712.625	31,874.135	Target 1
1	A1	3	60.627	1,331,073.750	32,174.191	Target 1
1	A1	4	60.940	1,321,421.000	32,461.256	Target 1
1	A1	5	61.253	1,311,457.625	32,703.426	Target 1
1	A1	6	61.566	1,301,217.750	32,885.973	Target 1
1	A1	7	61.879	1,291,080.625	33,008.629	Target 1
1	A1	8	62.193	1,279,632.750	33,081.289	Target 1
1	A1	9	62.506	1,267,451.375	33,120.445	Target 1
1	A1	10	62.819	1,257,889.125	33,141.980	Target 1
1	A1	11	63.132	1,247,544.125	33,154.762	Target 1
1	A1	12	63.445	1,236,449.250	33,154.816	Target 1
1	A1	13	63.759	1,225,509.750	33,131.277	Target 1
1	A1	14	64.072	1,214,965.250	33,076.242	Target 1
1	A1	15	64.385	1,204,268.750	32,995.559	Target 1
1	A1	16	64.698	1,193,365.125	32,907.461	Target 1
1	A1	17	65.011	1,182,249.375	32,832.797	Target 1

Divergent Expression of the Norepinephrine Transporter (NET) in the LC following SPS

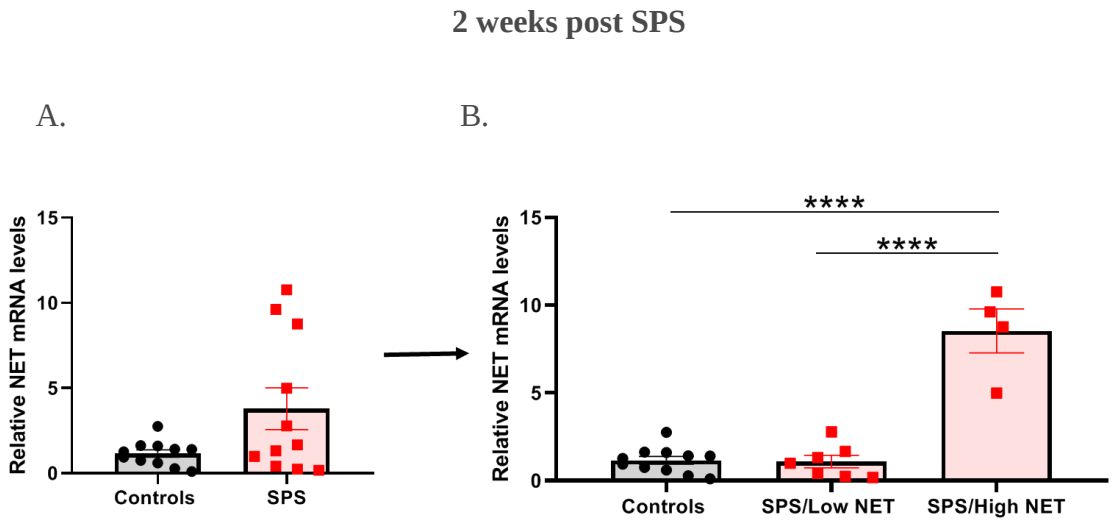


Figure 25. Changes in the Norepinephrine Transporters (NET) expression in the LC two weeks after exposure to SPS stressors. A. NET mRNA. B. NET mRNA divided into low and high groups. Low and high NET mRNA expression levels were based on mRNA expression level below the mean and mRNA expression level above the mean, respectively. Each point represents values for an individual animal. $^{**}p < 0.01$. For normality and log normality test the controls had a normal distribution while the SPS did not have a normal distribution. For both the Anderson-Darling and Kolmogorov-Smirnov (K-S) test, the control group passed normality test while the SPS group failed normality test. The mean was used to divide the SPS animals into two subgroups to visualize the large variety within the group. However, it doesn't give the result a different interpretation. The results show that there is variability between animals that received SPS compared to the unstressed controls.

4 weeks post SPS

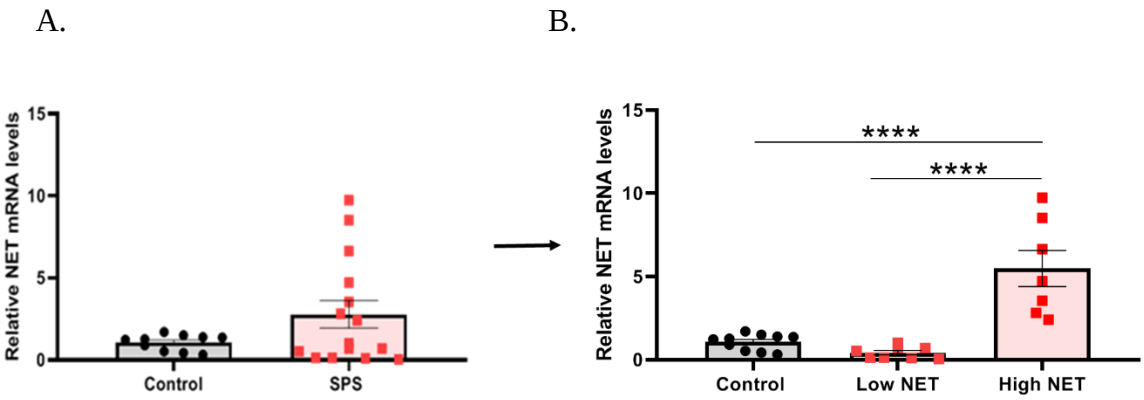


Figure 26. Changes in the Norepinephrine Transporters (NET) expression in the LC four weeks after exposure to SPS stressors. A. NET mRNA. B. NET mRNA divided into low and high groups. Low and high NET mRNA expression levels were based on mRNA expression level below the mean and mRNA expression level above the mean respectively. Each point represents values for an individual animal. ****$p < 0.0001$. For normality and log normality test the controls had a normal distribution while the SPS did not have a normal distribution. For both the Anderson-Darling and Kolmogorov-Smirnov (K-S) test, the control group passed normality test while the SPS group failed normality test. The mean was used to divide the SPS animals into two subgroups to visualize the large variety within the group. However, it doesn't give the result a different interpretation.

Two weeks after SPS, animals exposed to SPS stressors had divergent NET mRNA expression. Based on the variability in the SPS exposed group, we divided the SPS group to two subgroups, above and below the mean. One-way Anova revealed significance ($F(2, 19) = 57.78$; $P < 0.0001$) and Tukey multiple comparison tests showed significance ($p < 0.0001$) between low NET mRNA and high NET mRNA expressing animals. There was no significant difference between animals with low NET mRNA expression compared to the unstressed controls (Figure 25). In a different cohort of animals, divergent NET mRNA

expression was also observed four weeks after SPS (Figure 26), one-way ANOVA revealed significance ($F(2, 22) = 23.66$, $P<0.0001$). Tukey multiple comparison tests showed significance ($p < 0.0001$) between low NET mRNA and high NET mRNA expressing animals. There was no significant difference between animals with low NET mRNA expression compared to the unstressed controls.

These result indicate that animals in the SPS group had a divergent NET mRNA response to SPS and putting these animals into two subgroups is another way to visualize the divergent NET mRNA expression within the SPS group. The divergence NET mRNA expression in the LC has not been consistently observed in other mRNA expressions that we analyzed in the LC.

Effects of NPY on NET mRNA Expression in the Locus Coeruleus two weeks post SPS.

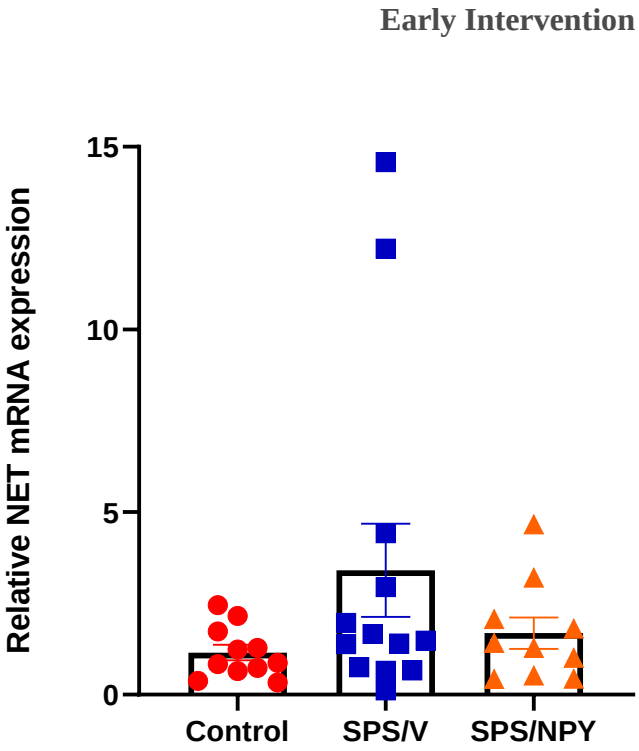


Figure 27. Effect of intranasal NPY on NET mRNA expression levels in the locus coeruleus two weeks after SPS.

In this cohort of animals, the divergence of NET mRNA expression in the SPS/V group is less compared to the divergence we observed for the cohorts in Figures 25 and 26. This is not surprising because they are of different cohorts and are all out breed. In addition to the previous experimental groups, here we had two groups of animals exposed to SPS stressors. One group received vehicle (SPS/V) and the second group was given intranasal NPY (SPS/NPY). NET mRNA expression of the SPS/NPY group exhibited responses similar to those of the unstressed controls, it was not significantly different from the SPS/V group. NPY tended to reduce the increase in NET mRNA expression in the SPS/NPY group two weeks after SPS.

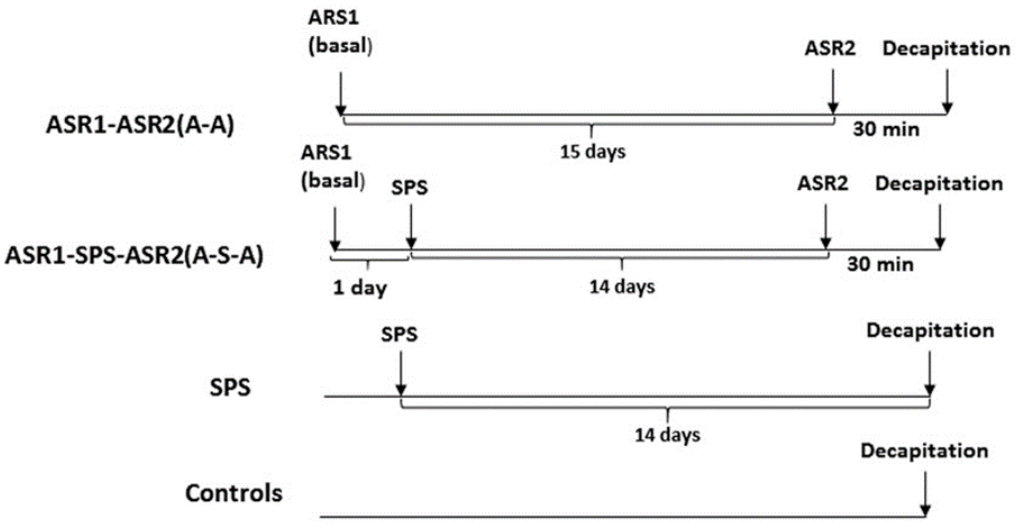
In summary, these results show that two weeks after exposure to SPS stressors, the NET mRNA expression of about 60% of the animals were similar to the control group while approximately 40% of animals had a higher NET mRNA expression. In a different cohort, four weeks after SPS, about 50% of the animals had NET mRNAs that were similar to the control group and the other 50% of the animals had a higher NET mRNA expression. Furthermore, early intervention with intranasal NPY tended to lower the increase in NET expression in a different cohort of animals two weeks after SPS.

The effects of SPS and ASR on Gene expression in the Medio basal Hypothalamus and

LC

Since hyperarousal is associated with activation of the NE/LC system [38, 154] and is elevated in PTSD [38] as well as by SPS [79, 81] we examined the effects of SPS triggered changes and acoustic startle response (ASR) on CRH, NPY and NET mRNA levels in both the mediobasal hypothalamus and the LC of the SPS animal model. ASR is LC specific because hyperarousal originates primarily in the LC which would enable the study of NET mRNA expression following ASR.

Schematic of experimental groups



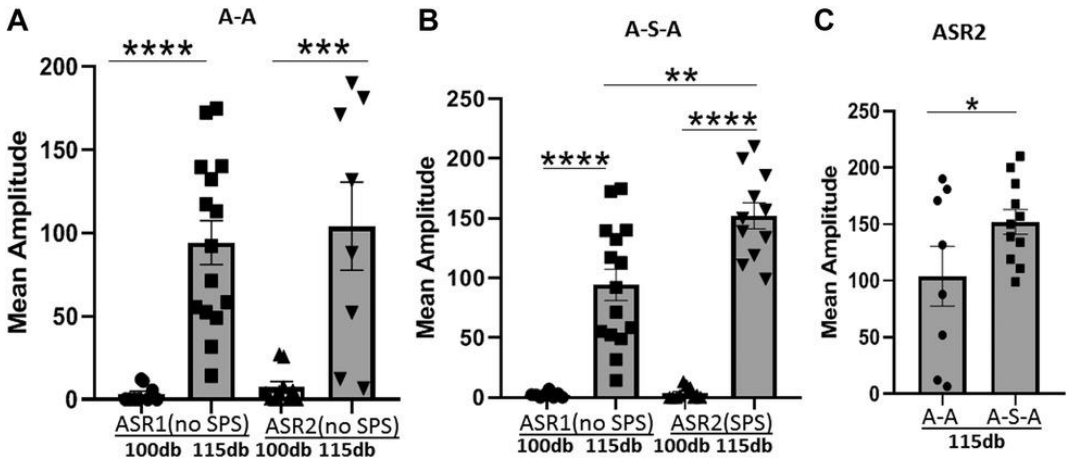


Figure 28. Mean amplitudes of startle. (A) ASR of A-A group (not exposed to SPS) at 100 and 115 db. (B) A-S-A group (exposed to SPS) at 100 and 115 db. For ASR1, data from A-A and A-S-A groups were combined. (C) ASR2 measured at 115 db for A-A and A-S-A groups, n = 8 for the A-A group and n = 11 animals for the A-S-A group. Means ± S.E.M. are shown. Each point represents values for an individual animal. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Acoustic Startle Response

ASR was measured at both 100 and 115 decibels. ANOVA analysis showed differences in ASR at 100 and 115 dB ($F(3,38) = 14.7$, $P < 0.0001$). Tukey comparisons of the means revealed that the startle response was much higher at 115 dB in both the A-A and A-S-A groups. The ASR of animals not exposed to SPS (A-A) was similar in both the first (basal ASR1) and the second (ASR2) test 15 days later at both 100 and 115 dB (Figure 28A). There were significant differences in ASR in the A-S-A group ($F(3, 40) = 43.4$, $P < 0.0001$). The startle response in the second test (ASR2) was significantly higher than for ASR1 at 115 dB (Figure 28B). The response to ASR2 at 115 dB was also greater in the stressed (A-S-A) compared with the unstressed (A-A) group (Figure 28C).

mRNA changes in the Mediobasal hypothalamus

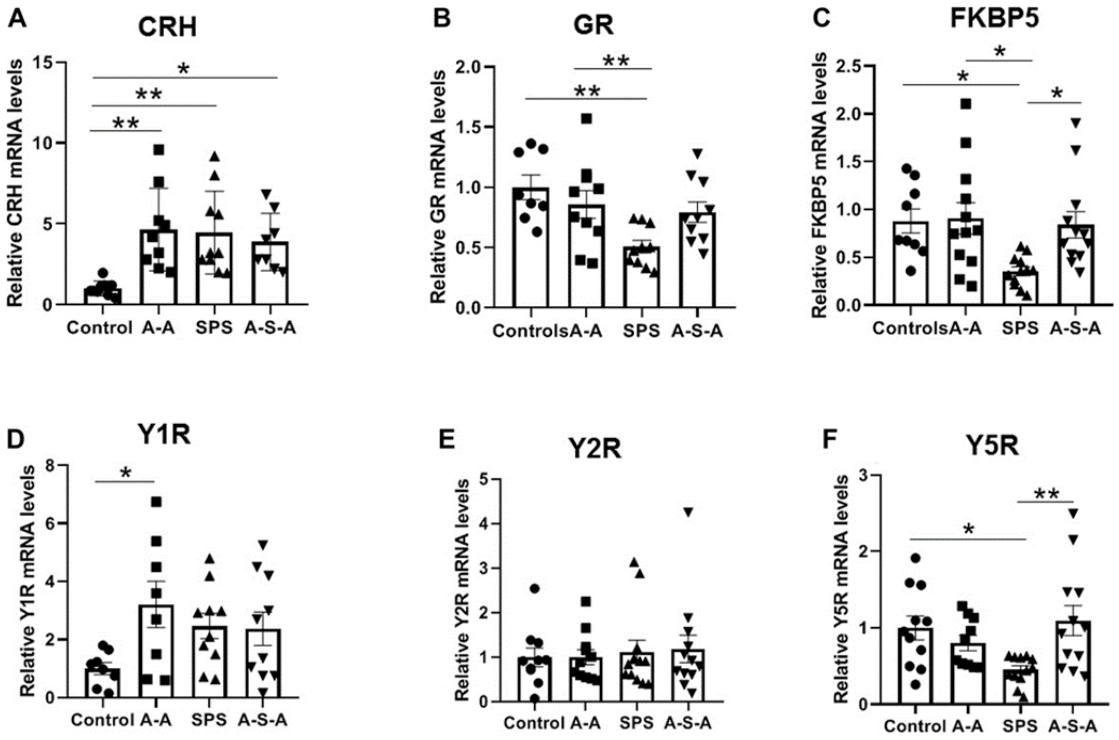


Figure 29. Changes in gene expression in the mediobasal hypothalamus. Relative mean ± SEM mRNA levels for CRH (A), GR (B), FKBP5 (C), Y1R (D), Y2R (E), and Y5R (F) are shown. Each point represents values for an individual animal. $^{*}P < 0.05$, $^{**}P < 0.01$.

The mRNA levels for CRH, GR, FK506 binding protein (FKBP5), and Y1R, Y2R, and Y5R receptor subtypes were measured in the mediobasal hypothalamus (Figure 29) to assess the possible influence of AS on PVN regulation with or without SPS exposure. CRH gene expression was altered ($F(3,32) = 6.19$, $P < 0.01$). CRH mRNA levels were higher than unstressed controls in all the groups, including the A-A group not exposed to SPS (Figure 29A). There were significant differences between the groups in GR and FKBP5 mRNA levels ($F(3, 34) = 5.13$, $P < 0.005$ for GR and $F(3, 41) = 4.390$, $P < 0.01$ for FKPB5). The mRNA levels for GR and FKPB5 in the A-A group were similar to controls. SPS reduced GR and FKBP5 mRNA expression (Figures 29B and C). Tukey comparison for both genes shows that the SPS group without AS is lower than in controls ($P < 0.01$, $P < 0.05$, respectively). However, there was no effect of SPS on GR or FKBP5 mRNAs in the group with basal ASR measurement (A-A vs A-S-A). One-way ANOVA analysis showed significance in gene expression for Y1R and Y5R ($F(3, 32) = 2.56$, $P < 0.05$ and $F(3, 41) = 4.33$, $P < 0.01$, respectively) but not Y2R (Figure 29D–F). Tukey comparisons showed that Y1R mRNA was elevated over the unstressed control group in the A-A group ($P < 0.05$) and Y5R mRNA levels reduced in the SPS group not exposed to ASR compared with the unstressed controls ($P < 0.05$).

mRNA changes in the Locus coeruleus

Figure 30

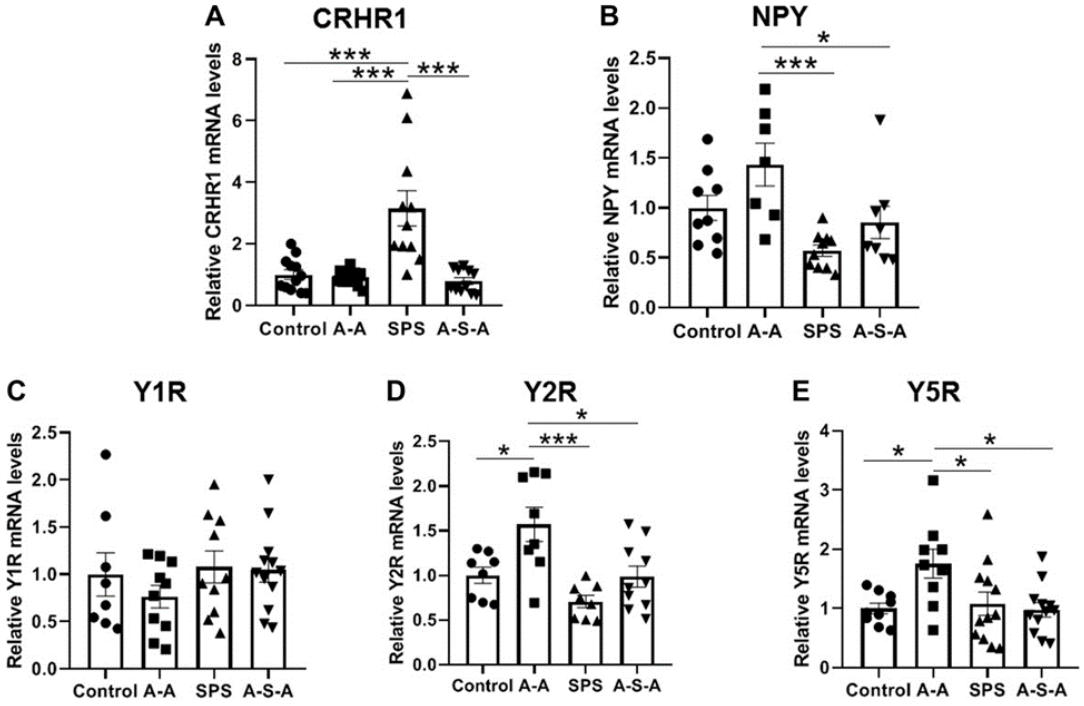


Figure 30. Changes in gene expression in the locus coeruleus. Relative mean ± SEM mRNA levels for: CRHR1 (A), pre-pro NPY (NPY) (B), Y1R (C), Y2R (D), and Y5R (E) are shown. Each point represents values for an individual animal. $^{*}P < 0.05$, $^{***}P < 0.001$.

There were significant differences in CRHR1 mRNA levels ($F(3, 41) = 14$, $P < 0.001$). CRHR1 was elevated ($P < 0.001$) only in the SPS group not exposed to ASR (Fig. 30A). One-way ANOVA analysis reveals differences in the expression of prepro-NPY mRNA levels ($F(3, 30) = 6.5$, $P < 0.01$). Tukey comparison revealed that the SPS and A-S-A were significantly lower than the mean of the A-A group ($P < 0.001$, $P < 0.05$, respectively) (Figure 30B). The mRNA levels were determined for the NPY receptor subtypes (Figures 30C–E). There were no differences in Y1R, but Y2R and Y5R were changed ($F(3, 30) = 7.952$, $P < 0.001$) and ($F(3, 38) = 4.1$, $P < 0.01$), respectively. Y2R and Y5R mRNAs were elevated in the A-A group over the unstressed controls ($P < 0.05$). The exposure to SPS (A-S-A group) reduced the mRNA levels for both Y2R and Y5R ($P < 0.05$).

These results revealed that acoustic startle (AS) alone triggered some changes through elevation of Y2R and Y5R mRNAs in the LC and CRH mRNA in the mediobasal hypothalamus was also elevated by either SPS, AS alone or both AS and SPS to similar levels. Current studies in the laboratory are focused on studying the effects of forced swim tests (FST) and elevated plus maze (EPM) in conjunction with changes in gene expression in the both the LC and mediobasal hypothalamus.

Divergent Expression of the Norepinephrine Transporter (NET) in the LC and relationship with Acoustic Startle

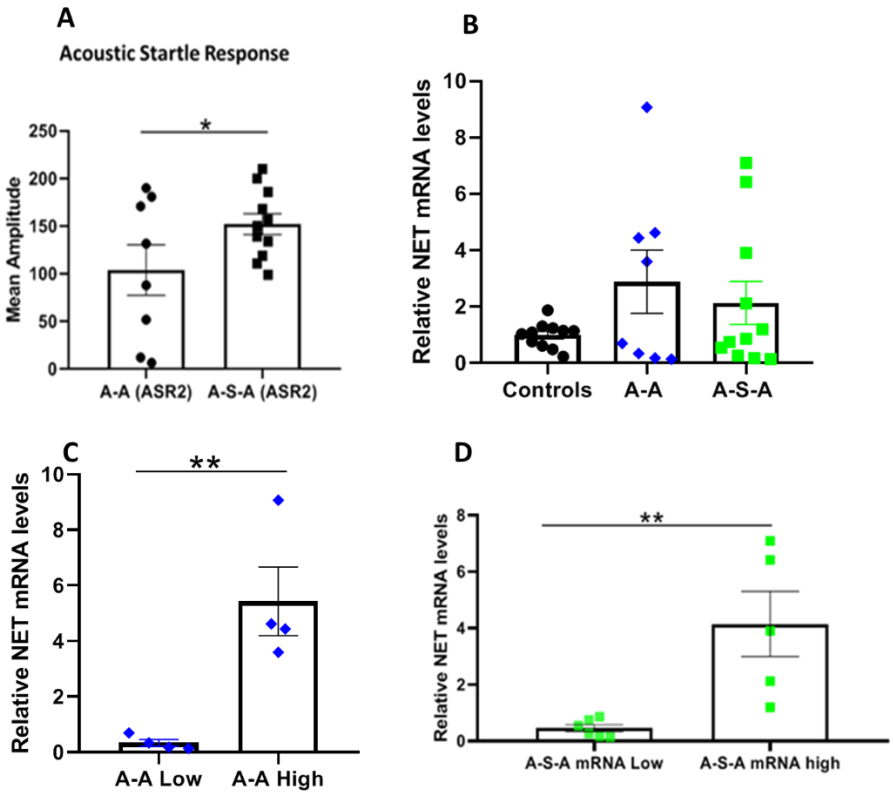


Figure 31. Acoustic startle response and NET gene expression in the locus coeruleus of the same cohort of animals. (A) ASR (B) NET mRNA levels in all groups (C) A-A group, NET mRNA is divided into low and high expression (D) A-S-A group, NET mRNA is divided into low and high expression. Low and high NET mRNA expression levels were based on mRNA expression level below the mean and mRNA expression level above the mean respectively. Each point represents values for an individual animal. $^{*}P < 0.05$, $^{**}P < 0.001$.

Correlation of the NET mRNA and ASR in the LC

Figure 32

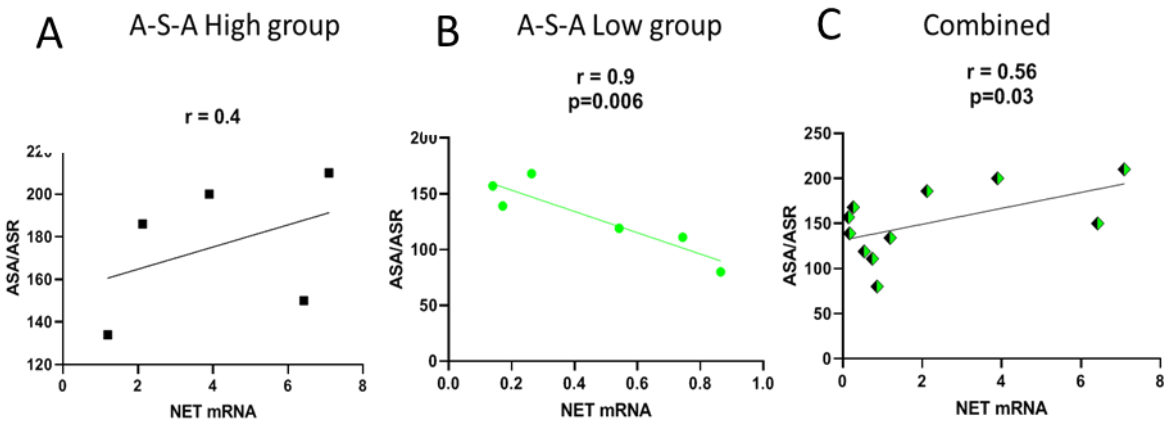


Figure 32. NET mRNA expression and ASR correlation. (A) High NET mRNA group Vs ASR (B) Low NET mRNA group vs ASR (C) NET mRNA of both groups combined and ASR. Low and high NET mRNA expression levels were based on mRNA expression level below the mean and mRNA expression level above the mean respectively. Each data point represents values for an individual animal.

ASR was higher in the A-S-A group. There was a divergence in the NET mRNA expression of animals that were exposed to acoustic startle alone (A-A) and both acoustic startle and SPS (A-S-A) in Figure 32A-D. t- test showed significance ($p< 0.01$) in A-A low vs high and in A-S-A low vs high (C & D). In addition, we observed a negative correlation of 0.9 and $p < 0.01$ between acoustic startle response and NET mRNA expression in the low subgroup of the A-S-A group (Fig 32A). There was not a strong correlation in the high subgroup of the A-S-A high group (Figure 32B). A correlation of 0.56 was observed when both subgroups were combined (Figure 32C). There was no relationship between ASR and NET mRNA in the A-A group (not shown). In conclusion, the SPS elicited changes in NET mRNA levels in the LC displayed a U-shaped relationship with the changes in acoustic startle. In addition, there was a negative correlation between changes in NET mRNA in the LC and ASR in the subgroup of animals that had lower NET mRNA expression level.

5.4.2 Norepinephrine transporter methylation analysis in the LC

Following the results on the correlation between changes in NET mRNA expression in the LC and hyperarousal in the A-S-A low group and coupled with the divergent NET mRNA response to SPS and acoustic startle we evaluated the methylation status of the NET promoter in the SPS treated animals compared to the unstressed controls. In addition, a study showed differential methylation at the NET promoter in ADHD patients. Two weeks after SPS we isolated both the left and right LCs and performed NET RT-PCRs using the right side of the LC. Based on the NET mRNA expression level result, in the SPS/V group we identified representative animals with low NET mRNA expression levels and those with high NET mRNA expression levels. Methylation analysis of the NET promoter using the left LC corresponding to the representative animals and the

unstressed control animals was performed. One-way ANOVA revealed significance ($F(2, 9) = 4.381$, $P < 0.05$) between groups.

Representative animals selected for promoter methylation

Figure 33

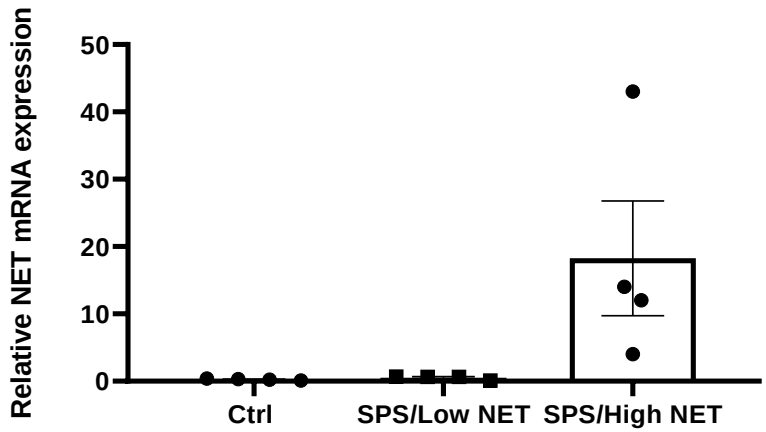


Figure 33. NET mRNA expression. Each data point represents values for an individual animal.

Two CpG regions in the NET promoter were studied slc6a2-1 and slc6a2-2

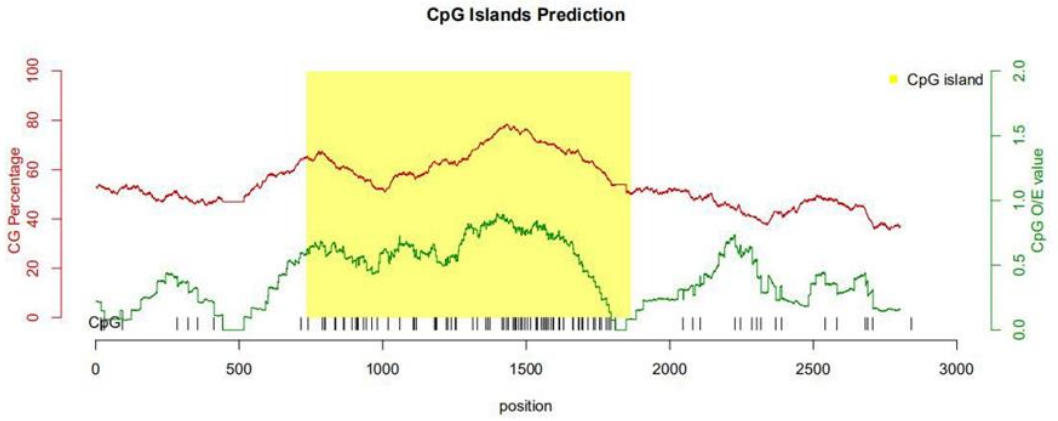


Figure 34. NET promoter methylation analysis. CpG region is shown (the red curve is the GC content, the green curve is the observed/expected (O/E) value and the yellow box represents the CpG Island). The ratio of observed to expected CpG is calculated as Obs /Exp CpG = Number of CpG * N / (Number of C * Number of G) where N = length of sequence. CpG locus sequenced relative to promoter start site are region1(slc6a2-1) -1199 to -947 and region 2 (slc6a2-2) -339 to -614.

NET Promoter Methylation

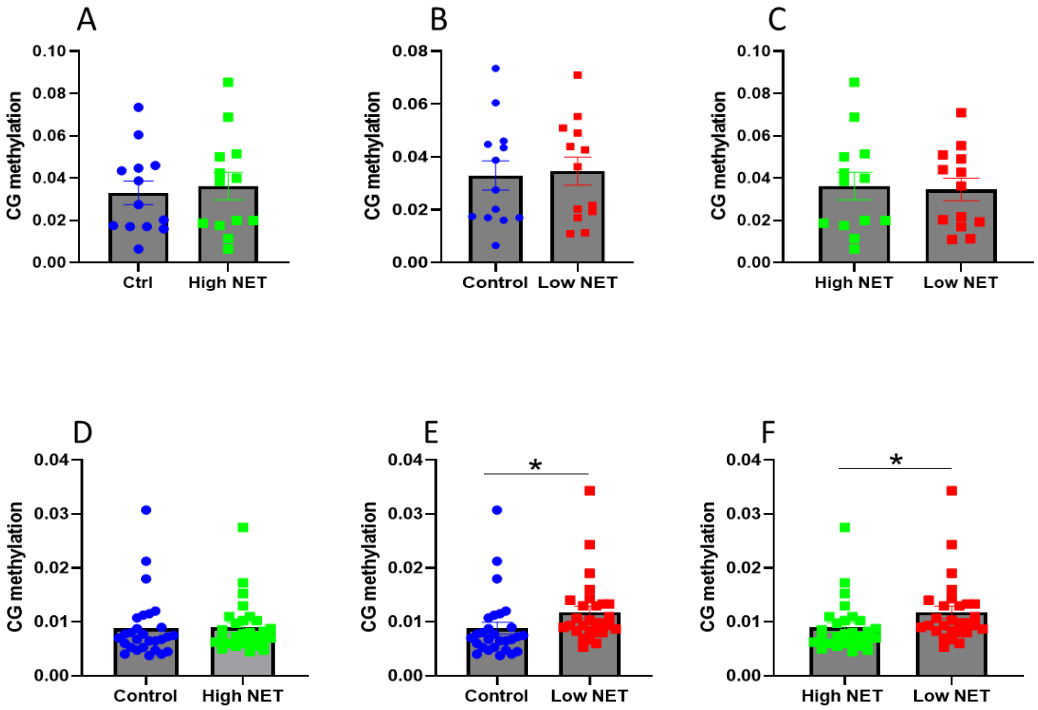


Figure 35. Region 1 (slc6a2-1) CpG methylation. (A) Control vs High NET (B) Control vs Low NET (C) High Net vs Low NET. Region 2 (slc6a2-2) CpG methylation. (D) Control vs High NET (E) Control vs Low NET (F) High Net vs Low NET. Each data point represents methylation sites. * $p < 0.05$

We found that representative SPS animals with low NET mRNA expression had increased methylation in CpG sites in region 2 of the promoter compared to either representative SPS animals with higher NET mRNA expression ($p < 0.05$) or the unstressed control animals ($p < 0.05$). There were no differences in methylation of CpG sites in region 1(Slc6a2-1).

Next, we wanted to evaluate NET protein expression in the SPS animal model. Chronic social stress was shown to increase NET mRNA and protein levels in the rodent LC and the projection regions of the hippocampus, frontal cortex and amygdala which could lead to reduced NE at their synapses [155].It has been suggested that the induction of NET is mediated by activation of the HPA axis. Chronic treatment with corticosterone for 21 days appeared to mimic this effect and increased NET mRNA and protein in the LC, and NET protein levels also in the hippocampus, frontal cortex and amygdala [156]. NE from the LC is involved in the encoding, consolidation, retrieval and reversal of hippocampal based memory [157]. NE was shown to regulate transcriptional control of long-term plasticity to gate the endurance of memory storage, the LC/NE system is key in orchestrating longevity of hippocampal-dependent memory [157].

We therefore evaluated the relative levels of NET protein in the LC and the hippocampus, a key target area for many of the effects of stress.

Reduced NET protein expression in the LC after SPS.

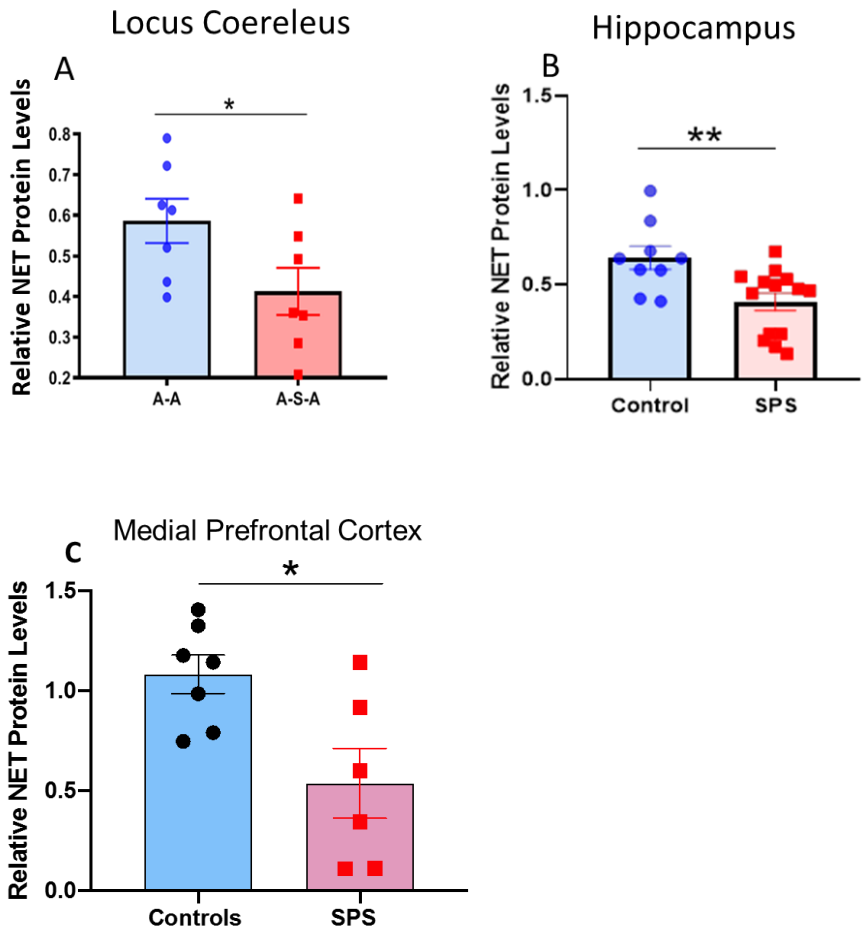


Figure 36. NET protein expression (A) Two weeks after SPS in the LC (B) Four weeks after SPS in the hippocampus (C) Two weeks after SPS in the medial prefrontal cortex (mPFC). Each point represents values for an individual animal. $^{*}P < 0.05$, $^{**}P < 0.01$.

NET protein expression was evaluated in the LC of animals that received only acoustic startle (AA) and animals that received both acoustic startle and SPS stressors and left undisturbed for two weeks. NET protein expression of animals that received both acoustic startle and SPS were lower ($p < 0.01$) than the animals that received only acoustic startle. In the both the hippocampus and mPFC, efferent terminals for projections from the LC, NET protein was reduced compared to the group not exposed to SPS. However, in the hippocampus of the SPS group, about 30% of the animals had NET protein expression level below the mean while 70% of the animals had NET protein expression levels above the mean and similar to the unstressed controls. A t-test revealed significance ($p < 0.01$) between the unstressed controls and the SPS group.

Based on this results we speculate that reduced NET protein in the LC could mediate exaggerated noradrenergic activation following a traumatic event and that reduced NET in the hippocampus could enhance memory consolidation of the trauma resulting in an increased chance of re-experiencing the traumatic event when a reminder presents. More studies are ongoing to further ascertain if these speculations are indeed correct.

Discussions

Development and progression of anxiety symptoms and the effects of Intranasal NPY treatment

Our studies show that anxiety symptoms worsen with time and would require doubling the dose of intranasal NPY for effective treatment (Figure 12). Intranasal infusion with 300 µg/rat reverses the SPS-elicited depressive like behavior in the FST. In our studies, the FST represented an element of re-experiencing because forced swim in the same cylinders was one of the SPS stressors. The implication is that re-experiencing could lead to potentially developing coping mechanisms to the forced swim test confounding the effects from SPS. Enhanced immobility on the FST has been interpreted as representing depressive/despair behavior or more accurately, shift from active to passive coping behavior [158]. FST has also been used to show the ability of ketamine to alleviate symptoms of depression [159]. It is also important to note that there have been considerations on whether the forced-swim test is a good model for depression in people. Furthermore, tests that accurately measure specific symptoms of depression, such as lack of interest in a task that used to be enjoyable could be more predictive of depressive-like symptoms.

NPY antagonizes the responses to stress-elicited elevation in CRH [160, 161], therefore activation of Gi by NPY would inhibit the CRH triggered elevation of cAMP by Gs. The Sabban laboratory has shown that early intervention with NPY delivered to the rat brain by intranasal infusion reduces the SPS-triggered activation of the HPA axis, elevation of plasma corticosterone or ACTH, and induction of GR in the hippocampus [78, 81]. 300µg/rat of NPY reversed hyperarousal (Figure 15). The reversal in hyperarousal was sustained and evident a week after administration of the intranasal NPY (Figure 16). It is speculated that PTSD could be associated with progressive neuronal sensitization [162], however the

neuronal mechanisms is unclear. In neuronal sensitization PTSD patients could appear to be unusually responsive to stress in contrast to as being less responsive to stress as seen by models of glucocorticoid resistance. It is speculated that this sensitivity would lead to change in excitability of neurons but causes and effects of neuronal sensitization is still unclear. NPY could be acting to depress the post-synaptic potential of LC neurons, via pre-synaptic Y2 receptors on the soma of the noradrenergic neurons [63, 163].

The Sabban laboratory showed previously that intranasal NPY also inhibits SPS-elicited activation of the locus coeruleus-noradrenergic system and induction of CRH in the central nucleus of the amygdala [96]. These results reinforce the idea that the severity of PTSD symptoms will determine how treatment is given as a higher dose of NPY may be required at longer times after SPS. These findings reinforce the idea that therapeutic treatment with NPY, or other medications to reverse or improve PTSD symptoms, to be administered according to the degree of the severity of the disorder and the sooner the better.

NPY Receptors Subtypes

The NPY Y1R subtype is sufficient in mediating the effects of NPY or [D-His^{26}] NPY to prevent the development of SPS elicited anxiety, social impairment and depressive-like behavior. We showed that intranasal administration of Y1R agonist [D-His^{26}]NPY can protect against the development of anxiety, depressive/despair behavior and impaired social interaction in the SPS rodent PTSD model [164]. However, the Y1R preferring agonist [Leu^{31}Pro^{34}]NPY was not effective for anxiety, although it inhibited the development of depressive-like behavior on the FST as well as sucrose preference [165]. By contrast, the Y2R agonist was not effective neither for anxiety nor for depressive-behavior. NPY displays 4-25-fold higher affinity for the Y2 receptor than for the Y1 receptor. The

affinity of [Leu^{31},Pro^{34}]NPY is 7-60-fold higher for the Y1 receptor when compared with the Y2 subtype [118].

Other factors that can influence the activity of agonists include binding affinities to receptors, concentration and stability. Our results show that activation of the Y1R by [D-His^{26}]NPY prevented the development of the symptoms of anxiety, social impairment and depressive-like behavior. Additional studies using specific receptor antagonist would further confirm that the effects of the agonist was mediated by the receptor.

The results on the effectiveness of Y1R agonist [D-His^{26}]NPY are consistent with earlier studies which have shown that anxiolytic-like effects of NPY are mediated via Y1 receptors while activation of Y2 receptors are anxiogenic. For example, Y1R antisense treatment of animals resulted in anxiogenic-like behavior on the EPM [117]. The Y1R preferring agonists [Leu^{31}Pro^{34}]NPY and [D-His^{26}]NPY were shown previously to have an anxiolytic-like effect in animals in the plus maze [74, 119, 166]. However, these results were in unstressed animals and not after exposure to traumatic stress. Here, single intranasal infusion of [D-His^{26}]NPY right after the traumatic stress was effective even two weeks afterwards, a time when SPS triggered anxiety is more severe [164].

In Y1R−/− mice, anxiety related parameters, such as distance travelled in the central area of an open field or open arm entry on the EPM were increased compared to the wild type. Interestingly, the anxiety-like phenotype in the Y1R mutant mice was dependent on several factors including circadian rhythm and prior stress exposure [167]. The icv administration of NPY to Y1R mutant mice did not produce anxiolytic-like effect in the EPM observed in WT mice, suggesting that the Y1R was needed for the anxiolytic effects [168]. Previously, we showed that Y1R preferring agonist [Leu^{31}Pro^{34}]NPY was effective in preventing development of depressive-like symptoms following the exposure

to SPS [165]. However unlike [D-His^{26}]NPY, it was not effective to thwart development of anxiety behavior. This could be due to [Leu^{31}Pro^{34}]NPY also activating Y5R [169]. [D-His^{26}]NPY has about 18 fold higher affinity for human Y1R than for Y5R and is 375 times more selective for rat Y1R than for Y5R to inhibit forskolin stimulated cAMP production [103].

Table 9. NPY Receptor Affinity

Receptors	Y1	Y2	Y4	Y5
NPY (nM)	0.28±0.06	1.2±03	ND	1.5±0.1
[D-His26]NPY (nM)	2.0±0.3	29.0±4.7	20.1±1.9	34.6±3.8

Adapted from Mullins et al., 2001[103] .

Alternatively, [D-His^{26}]NPY may be more stable, due to the presence of its D-amino acid [170]. This could make it more efficient in reaching different areas of the brain. The molecular mechanism of attenuation of traumatic stress sequelae by early intervention with intranasal NPY or Y1R agonists remains to be clarified.

Interestingly, we found that the [D-His^{26}]NPY was more effective than NPY to prevent the development of immobility on the FST. This could be due to the ubiquitous binding of NPY with high affinity and less selectivity for receptor subtypes or perhaps due to the greater stability of [D-His^{26}]NPY. NPY can act as a resilience factor by impairing associative implicit memory after stressful and aversive events, as evident in models of fear conditioning, presumably via Y1 receptors in the amygdala and prefrontal cortex [171]. Y1R deficient mice have significantly elevated immobility on FST compared to wild type animals suggesting that Y1 receptor agonist might have anti-depressive effects. However, they also displayed anti-depressive related response to fluoxetine, an anti-depressant [168].

In addition Y1 receptor stimulation attenuates the somatic signs associated with nicotine withdrawal making NPY or Y1 receptor agonists, [D-His^{26}]NPY potentially an effective treatment for symptoms associated with the discontinuation of tobacco smoking [171].

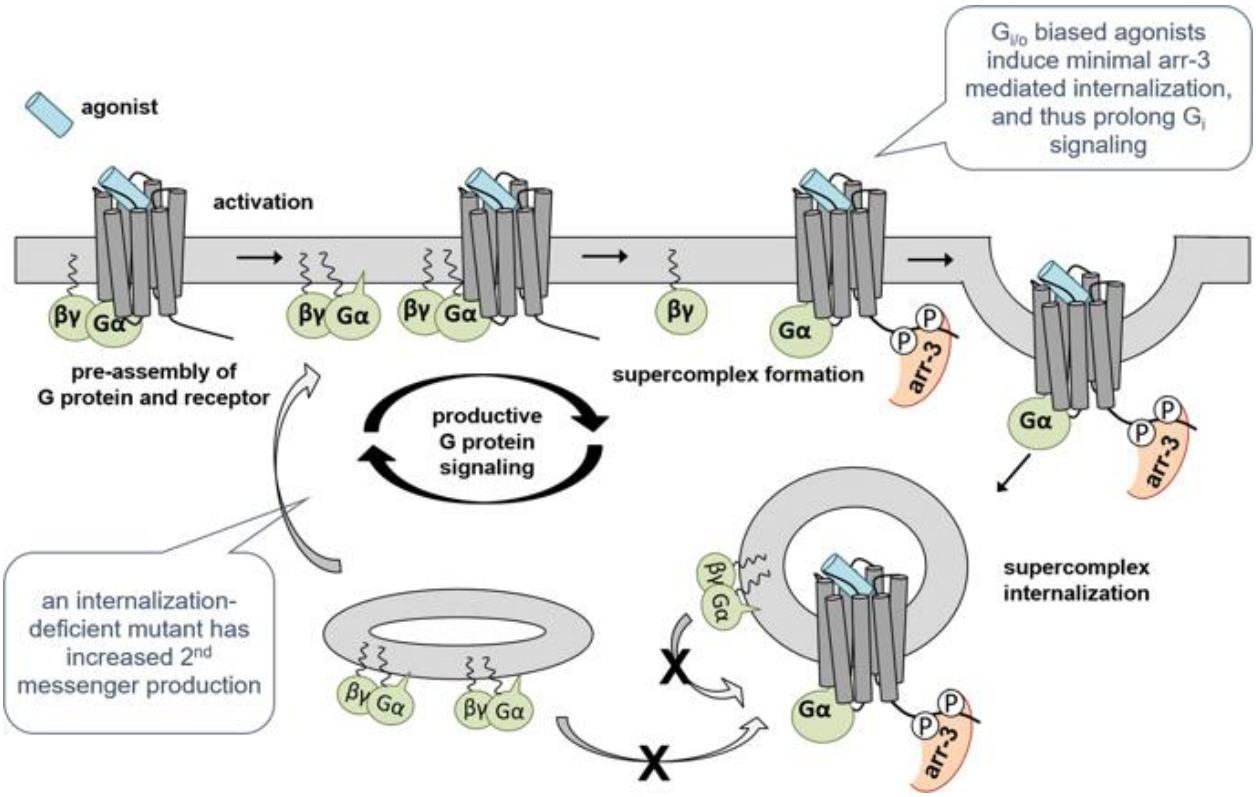
[D-His^{26}]NPY prevented development of the impaired social interaction (Figure 21) triggered by SPS. Its effects on hyperarousal need to be investigated further. There were large variations in the ASR of the SPS/V group (Figure 22), such that it is difficult to conclude whether or not [D-His^{26}]NPY was effective.

By contrast to Y1R agonist, the Y2R agonist NPY (3-36) did not prevent the anxiety or depressive-like behavior observed one week after SPS (Figure 20). In neurons that contain NPY, the Y2 receptor is mostly located presynaptically and negatively regulates NPY release [65, 172, 173]. The icv injection of Y2R-preferring agonist resulted in anxiogenic effect in the plus maze and social interaction tests [115, 116, 166]. Furthermore, the Y2 receptor knockout mice show less anxiety and more anti-depressive related behaviors [112, 174, 175]. However, results from the use of Y2R agonists have been diverse and depended on task and region [76, 176]. In contrast to our studies, the Y2 receptor preferring agonist NPY(3–36) did not attenuate the SPS elicited anxiety, as rats treated with the NPY(3–36) had similar anxiety levels as the vehicle treated group. In a previous study, a selective Y2R agonist was anxiogenic in the social interaction test when injected into the amygdala [115, 116]. While we have not examined the Y2R agonists for social interaction following SPS, in this study we found that [D-His^{26}]NPY blocked development of anxiety-like responses in the social interaction test (Figure 21) two weeks after SPS. Several studies have shown that NPY in the basolateral amygdala plays an important role in reducing anxiety-like behaviors, impairments in social interaction test and in fear responses [113, 115, 116, 177]. Based on the findings of other research groups and our studies, Y1R is possibly a key mediator

of NPY's protective effects on anxiety and depressive-like symptoms emanating from exposure to severe stress. Activation of the Y1R is sufficient to protect against the development of anxiety, impairments in social interaction and depressive/despair-like behaviors. [D-His^{26}]NPY was especially effective compared to [Leu^{31}Pro^{34}]NPY. It was even superior to NPY as an early intervention therapy to prevent the development of depressive-like symptoms and may have great therapeutic potential.

Furthermore, it is worthy to note that although it iswhether known that Y1, Y2 or Y5 receptor agonists can act as biased ligands, a more recent study showed that substitution of position Q34 of neuropeptide Y to glycine (G34-NPY) demonstrated selectivity over all YR subtypes [178] and displayed a significant bias towards activation of the Gi/o pathway and enhanced signaling over recruitment of arrestin-3 (Figure 37). G-protein coupled receptor (GPCR) signaling is a ligand-receptor-transducer complex that leads to a conformational state of the receptor. When stabilized by a selective biased ligand it promotes interaction with specific transducers such as G-protein or β-arrestin to evoke selective non canonical cellular signaling responses [179]. The advantage is that it provides opportunity to direct a Y1R agonist for more therapeutic potentials while minimizing off target or side effects.

Figure 37 - Biased ligand G ptrotein signaling



Adapted from Kaiser et al., 2020 [178]**.**

Figure 37. Proposed mechanism of Y1R biased ligand G protein signaling

Gene expression changes in the LC and mediobasal hypothalamus after SPS

Changes in the NPY and CRH mRNA expression were observed in the LC and mediobasal hypothalamus two weeks after SPS. Gene expression of CRHR1, the receptor subtype expressed in the LC, was previously shown to be elevated in a subset of animals one week after SPS [96]. Here, significant changes in CRHR1 persist and are especially robust after 2 weeks.

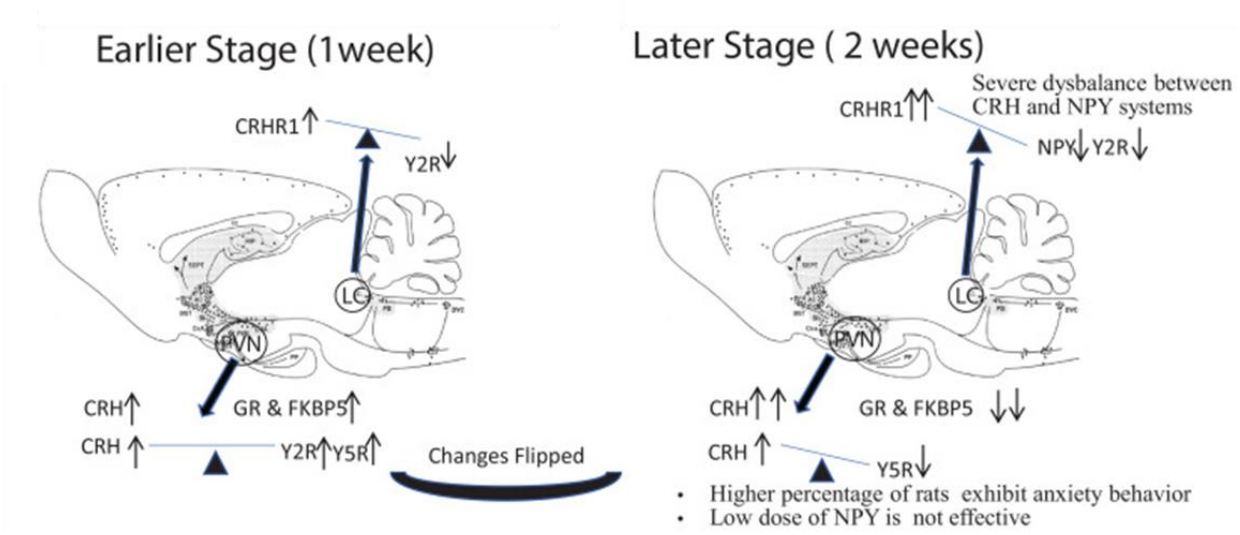


Figure 38. Summary of gene expression in the LC and mediobasal hypothalamus

There is a progressive dysbalance between the CRH and NPY systems in the LC as increased gene expression of CRHR1 (Figure 23) is accompanied by a sustained reduction of Y2R and NPY (Figure 23). Increase CRHR1 gene expression can enhance the ability of the LC to respond to the "pro-stress" CRH relative to the buffering NPY input in the weeks following SPS stressors. CRH afferents, arising primarily from the amygdala, PVN, Barrington's nucleus and nucleus paragigantocellularis project to the LC and provide a neuroanatomical pathway for an interaction between CRH and LC/NE neurons [180]. CRH drives the high tonic state of LC neuronal activity while simultaneously decreasing phasic firing events in the LC [181, 182]. CRH projections from the central nucleus of the amygdala to LC promotes an anxiogenic response, which is mediated by CRHR1 receptors [33].

Microinjections of NPY as well as agonist for Y2R, but not Y1R, into the vicinity of the LC, had anxiolytic effects on the EPM [76]. Exogenous NPY, possibly via Y2R, could depress the post-synaptic potential of LC neurons and potentiate the inhibitory effects of NE on the cell somata, thus reducing the firing of LC neurons [163]. SPS-triggered changes were observed in LC in several members of the NPY system, including NPY and Y2R. NPY is co-expressed with NE in 20–40% of LC neurons and projects to many brain regions including the hippocampus and cerebral cortex [183, 184]. Y2R is primarily a presynaptic receptor. It is involved in attenuating release of NE, as well as GABA. Sustained reduction of Y2R expression would be associated with down regulation of presynaptic inhibition, and over-activation of noradrenergic systems [82]. However, this is contrary to the study that showed less anxiety and more anti-depressive related behaviors in the Y2R knock out mice.

In contrast to the consistent elevation of the CRH gene expression a flip in the regulation of GR and FKBP5 gene expression between one and two weeks post-SPS

stressors was observed [164]. The mRNAs for GR and FKBP5 were increased over levels in unstressed rats in the early stage seven days after the SPS [78] 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 [26]. When FKPB5 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. FKBP5 is a co-chaperone of hsp90 that regulates glucocorticoid receptor (GR) sensitivity. When FKBP5 is bound to the receptor complex, cortisol binds with lower affinity and nuclear translocation of the receptor is less efficient. Cortisol binding allows efficient nuclear translocation of GR. FKBP5 mRNA and protein expression are induced by GR activation via intronic hormone response elements providing an ultra-short feedback loop for GR-sensitivity [24]. The PVN receives NPY mainly from the hypothalamic arcuate nucleus which innervates numerous PVN-CRH neurons [185] acting most probably via Y1R, Y2R and Y5R, Gi/Go coupled receptors [186]. 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 [187]. In unstressed rats, icv infusion of NPY into the brain increased CRH mRNA levels, predominantly via Y1R [188]. 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 [81, 85].

We found that SPS triggered 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 (Figure 24). Since the functions of Y1R and Y5R on anxiety behavior are similar [119] and higher levels of both receptors are associated with reduced anxiety [189], 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 of Y1R from Y5R expressing neurons caused an increase in anxiety behavior [190]. 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 (Figure 24). The Y2 receptor subtype was markedly increased, but only at one week following the SPS (Figure 24). Potential Y2R signaling may be anxiogenic, since as an autoreceptor its activation leads to a reduction in NPY release via a negative feedback loop [191].

Gene expression changes in the LC and mediobasal hypothalamus after acoustic startle and SPS

Analysis of changes in gene expression in the LC revealed that acoustic startle (AS) by itself was sufficient to trigger some changes, specifically elevation of Y2R and Y5R mRNAs (Figures). In the mediobasal hypothalamus, CRH mRNA was also elevated by either SPS or AS alone to similar levels (Figure 29). Exposure to AS appeared to dampen some of the response to SPS and when combined with SPS (A-S-A group) there was no further increase in CRH mRNA (Figure 30). It remains to be determined why the responses were not additive. There could be a threshold effect. Alternatively, if they both utilize a similar downstream mechanism, then the combined response would not be additive. The SPS-triggered reduction of GR and FKBP5 was not observed in the group (A-S-A) previously exposed to AS. In the LC, rats pre-exposed to AS did not display the SPS-triggered rise in CRHR1, which needs to be further investigated.

We speculate that the AS-triggered changes in expression of NPY receptors may be involved in dampening the responses to SPS. CRH from the PVN of the mediobasal hypothalamus is crucial for initiating HPA response to stress. As previously observed, SPS triggers sustained elevation of CRH gene expression 1 week following SPS [78]. Here we show that CRH mRNA remains high 2 weeks after exposure to SPS stressors and that AS is sufficient to trigger elevated CRH gene expression in the mediobasal hypothalamus. The PVN receives NPY mainly from the hypothalamic arcuate nucleus, and it has been demonstrated that numerous NPY-containing axons innervate the PVN–CRH–containing neurons (The Y1R, Y2R, and Y5R receptor subtypes are expressed in the mediobasal hypothalamus, [88, 186]. This pathway is essential in the regulation of neurosecretion of CRH in response to mild stress such as AS, allowing a possible crosstalk between these two neuropeptide systems [85, 90]. Both Y1R and Y5R are postsynaptic receptors, although Y5R

is less abundant compared with Y1R. The Y5R can be co-expressed with Y1R receptor [85, 88]. The importance of NPY and Y1R in the hypothalamus has previously been recognized for its involvement to promote adaptive stress responses in the predator scent model of PTSD [192-194].

Following a traumatic event, CRH from hypothalamic and extra hypothalamic sources, especially the amygdala, changes the pattern of LC firing to one promoting cognitive flexibility [181]. By acting via CRHR1, it increases c-Fos and tyrosine hydroxylase expression and neuronal discharge in the LC, with subsequent NE release in cortical and subcortical structures [195, 196]. CRH levels are elevated in the CSF of PTSD patients [197, 198]. In depression, a comorbid symptom in many PTSD patients, CRH levels become chronically elevated in some patients. Sustained elevated CRH was observed in the CSF and limbic areas of the postmortem brain of depressed patients and patients who were victims of traumatic experience as children [199]. It has also been demonstrated that icv infusion of CRH to rats led to pronounced, dose-dependent enhancement of the AS reflex [200]. CRH leads to the release of adrenocorticotropic hormone from the pituitary and subsequent elevation of glucocorticoids (cortisol in humans, corticosterone in rats) in the bloodstream. GR is activated by glucocorticoids and this facilitates the feedback inhibition of the HPA axis. Dysregulation in GR function within brain regions that are essential for crucial regulation and termination of the HPA axis activity might be responsible for some of the PTSD-related features [201, 202]. FKBP5 itself is a GR-response gene and modulates GR function by preventing the appropriate binding and shuttling of GR to the nucleus leading to glucocorticoid resistance [202, 203]. FKBP5 polymorphisms and promoter methylation status have been associated with the prevalence of developing PTSD [25]. In this part of the study, there were no changes found in mRNA levels for GR and FKPB5 in the A-A group of rats.

These results may indicate that the physiological response to AS, which is considered a mild stress, leads to rapid activation of CRH gene expression without significant modifications of the GR–FKBP5 regulatory mechanism. However, it is plausible that the changes in mRNAs for these genes might occur later since they are involved in feedback regulation.

The Y1R mRNA levels were significantly increased 30 min after testing for ASR2 in the A-A group (Figure 29) when compared with the unstressed controls. This likely leads to the activation of NPY neurotransmission via postsynaptic Y1R receptors to PVN to balance the increase of CRH gene expression by AS and to provide proper termination of responses to the mild stress. Although rats from the A-A group did not show alterations in Y5R mRNA levels 30 min after the ASR2 test, rats exposed to AS and SPS stressors (A-S-A) had Y5R mRNA levels higher than the SPS group (Figure 29). No significant changes in Y2R mRNA levels were observed among the groups.

Expression of stress related genes associated with response of the LC to SPS was changed by AS and SPS. CRH drives the high tonic state of the LC neuronal activity while simultaneously decreasing phasic events in the LC [181, 182] CRH projections from the central nucleus of the amygdala to the LC promote an anxiogenic response mediated by CRHR1. This increased tonic LC-NE activity is necessary and sufficient for the stress-induced anxiety and aversion [33]. We previously showed that CRHR1 mRNA levels are increased in some animals 1 week after exposure to SPS [96]. We found the elevation of CRHR1 mRNA levels 2 weeks after exposure to SPS (Figure 30) which is consistent with our previous study [82, 96].

However, to our surprise, in the A-S-A group that had ASR1 measurement before exposure to SPS and ASR2 measurements 2 weeks after SPS, CRHR1 mRNA levels were

similar to the unstressed control and to the A-A group. This would suggest that ASR could be dampening the response to SPS stressors. This speculation can be drawn from the elevation of Y2R and Y5R observed in response to ASR alone. However, since we do not have measurements of gene expression at the time of administration of SPS (1 day after basal ASR) without ASR2, it is unclear how ASR might be dampening this effect. Elevation of glucocorticoids by exposure to ASR1 may be mediating these effects as administration of corticosterone 12 h prior to acute stress protected against the delayed behavioral and cellular effects of acute stress on the amygdala [204].

Y2R is a presynaptic receptor involved in regulating the release of NE. The angiogenic role for Y2R was demonstrated when administered into some brain locations or in transgenic animals [112, 172]. Microinjections of NPY and agonist for Y2R into the LC region had anxiolytic effects [76] and were sufficient to attenuate the postsynaptic potential of LC neurons resulting in the inhibitory effects of NE on the cell somata and decreasing the firing rate of the LC neurons [163]. In this study, NPY mRNA levels in the LC were significantly reduced 2 weeks after SPS and in the A-S-A group. Y2R mRNA expression was previously shown to be reduced in the LC 1 week after exposure to SPS stressors [96]. We observed a sustained reduction of Y2R mRNA 2 weeks after exposure to SPS. AS by itself led to elevated levels of Y2R and Y5R gene expression in the LC of the A-A group but no changes in the Y1R gene expression was observed. Overall, SPS and AS by themselves triggered changes in gene expression in the LC and the mediobasal hypothalamus two weeks after exposure to stressors. Further studies are required to understand how AS regulates the expression of specific stress-related genes.

Divergence in the norepinephrine transporter (NET) mRNA levels after SPS and ASR

Our studies demonstrated a divergent NET mRNA response following either SPS or acoustic startle response and an association between hyper arousal symptoms originating in the LC and the divergent expression of NET mRNA in the LC in an animal model of PTSD. Two weeks after SPS the NET mRNA expression of about 60% of the animals were similar to the control group while approximately 40% of animals had a higher NET mRNA expression. In a different cohort about 50% of the animals had NET mRNAs that were similar to the control group and the other 50% of the animals had a higher NET mRNA expression (Figure 25). This divergent NET response was also observed after animals were exposed to acoustic startle (Figure 31). The animals that showed increased ASR were found to have higher NET mRNA levels. In addition, there was a negative correlation between changes in NET mRNA in the LC and ARS in the subgroup of animal that had lower NET mRNA expression level. Furthermore, we found that SPS has an effect on NET promoter methylation (Figure 35). The NET promoter of the subgroup of SPS animals with lower NET mRNA expression had higher methylated CpG sites in a proximal CpG island on the rat promoter region compared to SPS animals with a higher NET mRNA expression.

To our knowledge, this is the first observation in a PTSD animal model that is suggestive of a potential stress resilience or susceptible mechanism by the norepinephrine transporter. A recent study showed differential DNA methylation levels in the NET promoter between patients with attention deficit disorder (ADHD) and healthy controls (HC) [59]. DNA methylation is an epigenetic mechanism in which a methyl group is added to cytosine in cytosine–phosphate–guanine sites (CpG) and has received increased attention as a possible modulator in psychiatric disorders [205]. It can interfere with the

binding of transcription factors while methyl transferase activity can result to gene repression. In postural tachycardia syndrome (POTS) patients, change in NET expression is attributed to increased binding of the repressive MeCP2 regulatory complex, in association with an altered histone modification composition at the promoter region of NET [206]. Differential NET protein expression was associated with specific histone modifications in healthy individuals and POTS. Increased levels of circulating norepinephrine (NE) was observed in POTS patients as a result of impairment in the clearance of NE by NET from the synapse [207]. A mutation in the norepinephrine transporter gene resulted in loss of function and impairment of synaptic norepinephrine clearance leading to excessive sympathetic activation [208] as seen in the POTS patients.

Heightened arousal and reactivity, a core symptom of posttraumatic stress disorder (PTSD) originates in the LC [154]. The association of startle response with divergent NET mRNA expression and differential methylation in region of the NET promoter is suggestive of a possible resilience and susceptibility mechanisms. Furthermore, we observed a reduction in total NET protein two weeks after SPS compared to control animals in the LC and hippocampus. A PET study showed a positive correlation between vigilance and norepinephrine transporter availability in the LC of patients with PTSD [58] and in both the LC and thalamus in depressed patients [209]. It has also been shown that chronic cold stress increased the proportion of plasmalemmal NET in stressed rats compared to that in controls [210]. Chronic administration of corticosterone to rats caused chronic stress, increasing both NET mRNA and protein levels in the rat LC as well as LC projection regions such as the hippocampus, frontal cortex, and amygdala [156]. There is increased central nervous system responsiveness to noradrenergic signaling in individuals with a history of traumatic exposure [211]. The level of NET mRNA transcription can change in response to adrenergic activation after a traumatic event. Exaggerated

adrenergic activation following a traumatic event can enhance memory consolidation of the trauma resulting in an increased chance of re-experiencing the traumatic event when a reminder presents [212].

Adrenergic α_2 and Y2R receptors can also affect the concentration of NE in the synapse. They are presynaptic receptors and control the release of NE, inhibition of these presynaptic receptors can cause an increase in NE synaptic levels. Therefore, it is possible that down regulation of NET could lead to an upregulation of the α_2 receptors which negatively controls the release of NE.

Changes in the density and function of pre- and postsynaptic ARs results from long term inhibition of NET by antidepressants such as desipramine [213]. NET also has a higher affinity than the dopamine transporter (DAT) for dopamine (DA) and can transport DA as well as norepinephrine (NE) [214, 215].

NET-deficient (NET-KO) mice behave like antidepressant-treated mice and mRNAs encoding the alpha(2A)-adrenergic receptor and the alpha(2C)-adrenergic receptor are up-regulated in the brainstem [216]. NET-KO mice display higher resistance to convulsions [50] and are supersensitive to psychostimulants [49]. NET has also been a target for the action of many drugs used to treat major depression [51]. Specific NET inhibitor nisoxetine normalized the prepulse Inhibition deficits in dopamine transporter knockout (DAT KO) Mice [217].

NET Protein Expression

Reduced NET protein in hippocampus and medial prefrontal cortex after SPS compared to control animals found in this study is in line with the finding that NET availability in the LC and limbic brain regions from PET scans of PTSD patients was decreased compared to healthy controls [218]. These studies also found a positive correlation between vigilance and NET availability in the LC in patients with PTSD [218]. Altered NET function was observed in patients with major depression, with elevated NET availability in the thalamic subregion connected to the prefrontal cortex. This was positively correlated with attention [209]. Reduced NET expression in LC is likely to be associated with increased LC neuronal activity, this remains to be determined. However, NET activity can be regulated not only by its total protein levels, but also by its plasmalemmal distribution. Chronic cold stress increased the proportion of NET on the plasma membrane in the rat prefrontal cortex [210].

The discrepancy between the changes in mRNA and protein might reflect a lag between transcription and translation or transport of the protein. Also SPS might be altering the turnover rate of the protein. Further studies with more projection areas and a more detailed time course could help to clarify this.

NET is also regulated by glycosylation. Carbohydrate units of glycoproteins are involved in controlling protein folding, stabilizing protein conformation, protecting against proteolysis, and regulating intracellular and surface trafficking [219]. Poorly or unglycosylated forms of NET are not directed to the surface and are retained in the cytoplasm [220, 221]. In addition, PKC activation induces phosphorylation of both serine and threonine residues in rat and human NETs, resulting in transporter phosphorylation and down-regulation [222].

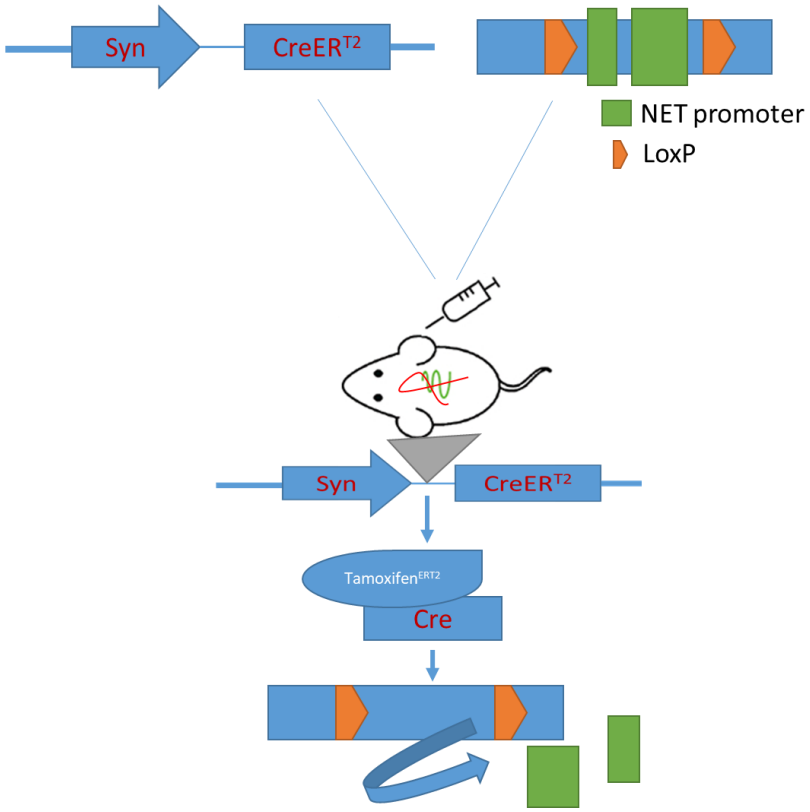
We demonstrate for the first time a divergent NET mRNA response to stress that can serve as a molecular basis for an in-depth study on the involvement of NET and the LC-NE system in predicting resilience or susceptibility in PTSD. These findings will facilitate in the identifications of biomarkers that will help in diagnosis and more effective treatments of PTSD symptoms in patients.

Limitations

Some limitations of the NET study would be addressed in future studies by increasing the sample size to further study the relationship between hyperarousal symptoms and time course in NET expression. In addition, to evaluate NET protein expression in the medial prefrontal cortex, amygdala, LC and hippocampus.

Possible future experiments to evaluate if NET is necessary and sufficient to attenuate hyperarousal response.

Possible future experiments to further decipher the role of NET in ASR would include pharmacological experiments inhibiting NET with reboxetine or other selective NET inhibition and then expose animals to SPS or acoustic startle to evaluate if down regulation of NET just before ASR will affect the ASR. However, because down regulation of NET could lead to an upregulation of the α2 receptors which negatively controls the release of NE, upregulation of mRNAs encoding the alpha(2A)-adrenergic receptor and the alpha(2C)-adrenergic receptor in the brainstem and upregulation of dopamine transporters. The precise conditional control of NET expression in vivo could be more suitable to evaluate the effect of NET on ASR. For example, the Cre/lox site-specific recombination system can allow the control of a specific gene activity in space and time in almost any tissue of the animal. To make the Cre/lox system inducible, ligand-dependent chimeric Cre recombinases, CreER recombinases, have been developed [223, 224]. They consist of Cre fused to mutated hormone-binding domains of the estrogen receptor. The CreER recombinases are inactive, but can be activated by the synthetic estrogen receptor ligand 4-hydroxytamoxifen (OHT), therefore allowing for external temporal control of Cre activity. A schematic of potential animal construct is shown below.



To make it more LC specific, cocktail of lenti-virus construct of CreER fused to the human synapsin promoter and a floxed NET promoter construct will be injected into the LC. This will knock down the expression of NET more precisely and specifically in the LC. Administration of tamoxifen will allow for conditional downregulation of NET before and after SPS or ASR. We would expect that this would lead to reduced ASR.

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