I, Sarah N Schmeltzer, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Neuroscience/Medical Science Scholars Interdisciplinary.

It is entitled:
The role of forebrain Neuropeptide Y in the regulation and development of PTSD-like behaviors

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Role of forebrain Neuropeptide Y in the regulation and development of PTSD-like behaviors

A dissertation submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

in the Graduate Program in Neuroscience
of the College of Medicine
by

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June 2010

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General Abstract

Posttraumatic Stress Disorder (PTSD) is a debilitating psychiatric syndrome evoked by trauma. Underlying mechanisms and neurobiological factors contributing to PTSD pathophysiology are still being understood. Recent studies implicate Neuropeptide Y (NPY), a 36-amino acid peptide transmitter. NPY is highly expressed in forebrain regions of the amygdala, prefrontal cortex and hippocampus, which show dysregulation in PTSD. In previous studies, we reported reduced CSF NPY levels in veterans with combat-PTSD compared to healthy volunteers and combat exposed veterans (Sah et al., 2009, 2014). Polymorphisms in the NPY gene are associated with stress susceptibility and coping. Investigating how NPY contributes to PTSD pathophysiology could lead to a better understanding of the disorder, and identify potential therapeutic and diagnostic markers for PTSD.

This dissertation investigates contributions of forebrain NPY to PTSD relevant behaviors using rodent models. Studies primarily focused on the medial prefrontal cortex (mPFC) and the amygdala, regions reported to malfunction in individuals with PTSD. Using pharmacological interventions, significant effects of infralimbic (IL) NPY were observed on extinction consolidation and retrieval of extinction. Functional modulation of excitatory-inhibitory neurotransmission and localization of NPY Y1 receptor on IL projection neurons revealed a pathway by which elevated IL-PFC NPY may modulate fear memory. Using tract-tracing studies, a novel NPY efferent pathway from the raphe nucleus to the IL cortex was identified. Since raphe-IL pathways are reportedly recruited in behaviors resulting from uncontrollable stress situations, this NPY circuit is particularly relevant to PTSD, a disorder stemming mostly from uncontrollable trauma.

To date studies on NPY in the amygdala have revealed fear and anxiety reducing effects, although site-specific studies are scarce. Our pharmacological and lentivirus
intervention approaches reveal complex, site-specific actions of NPY on fear. While NPY delivery into the basolateral amygdala, an area enriched in inhibitory Y1 receptors, reduced conditioned fear, NPY in the paracapsular areas enriched in GABAergic intercalated cells led to potentiation of conditioned fear and compromised extinction. Collective results from chapters 2 and 3 reveal region, site, and circuit-specific effects of NPY, questioning previous global intervention studies reporting fear and anxiety reducing actions of the peptide.

In humans, chronicity of trauma promotes worsened PTSD outcomes. In the last chapter, a rodent model of chronic versus acute traumatic experience on the development of fear and startle responses was characterized. NPY regulation in forebrain regions was assessed. A history of chronic stress worsened acquisition of fear and conditioned fear expression compared to an acute stress cohort. NPY was significantly attenuated in the hippocampus. Potentiated neuronal activation (FosB/ΔFosB-like immunostaining) was evident in the dentate gyrus, BLA, and mPFC of chronically stressed animals suggesting recruitment of these regions.

This body of work demonstrates NPY can have profound, site specific effects on fear memory and stress-associated behaviors of relevance to PTSD. Future studies should adapt cell and circuit-targeted approaches. Our studies necessitate the investigation of regional differences in NPY expression in postmortem tissue and the extrapolation of CSF NPY to these differences to establish the relevance of NPY to PTSD.
Acknowledgements

There are so many people that I need to thank for helping me get where I am today. There are so many of you, in fact, that I may leave out a few by accident (please forgive me!). First, I would like to thank my mentor, Dr. Renu Sah for allowing me the opportunity to complete my dissertation in her lab. I have learned so much from you, and the skills I have gained, even the non-scientific ones, will help me greatly in the future. I know that I am a better scientist and person for having learned from you.

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work with and taking being the only guy in lab in stride. I know you will be successful in the
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an office for a little while), you are an excellent addition to our lab, and no one does lab
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awesome mentee and I hope we stay in touch, because I see awesome things in your future.

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Finally, I would like to dedicate this dissertation to my grandfather, Omer Schmeltzer, and my father-in-law, Louis “Padre” Gainey. You both supported and encouraged me on this journey, and I am sad that you are not here to see it end, because I know you would be there, proudly sitting in the front row.
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<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>ASR</td>
<td>Acoustic startle response</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BA</td>
<td>Basolateral nucleus of the amygdala, basal division</td>
</tr>
<tr>
<td>BDI</td>
<td>Beck Depression Index</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BL</td>
<td>Basolateral nucleus of the amygdala, lateral division</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral nucleus of the amygdala</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus of the Stria Terminalis</td>
</tr>
<tr>
<td>CaMKIIα</td>
<td>Calcium calmodulin kinase II alpha</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPS</td>
<td>Clinician administered PTSD Scale</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CeA</td>
<td>Central nucleus of the amygdala</td>
</tr>
<tr>
<td>CeM</td>
<td>Central nucleus of the amygdala, medial division</td>
</tr>
<tr>
<td>CeL</td>
<td>Central nucleus of the amygdala, lateral division</td>
</tr>
<tr>
<td>CES</td>
<td>Combat Exposure Scale</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic adenosine monophosphate response element-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRHBP</td>
<td>Corticotropin-releasing hormone binding protein</td>
</tr>
<tr>
<td>CRHR1</td>
<td>Corticotropin-releasing hormone receptor 1</td>
</tr>
<tr>
<td>CRHR2</td>
<td>Corticotropin-releasing hormone receptor 2</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CVS</td>
<td>Chronic variable stress</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus of the hippocampus</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal raphe nucleus</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>DWS</td>
<td>Delayed Win-Shift task</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>eEPSC</td>
<td>Evoked excitatory postsynaptic current</td>
</tr>
<tr>
<td>eIPSC</td>
<td>Evoked inhibitory postsynaptic current</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAM</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>FG</td>
<td>FluorGold</td>
</tr>
<tr>
<td>FKBP5</td>
<td>FK506 Binding protein</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
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<tr>
<td>GABA</td>
<td>Gamma-amino-butyric acid</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>GABAAR</td>
<td>GABA&lt;sub&gt;a&lt;/sub&gt; receptor</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-coupled inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated channel</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HFS</td>
<td>High frequency stimulation</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenocortical</td>
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<td>HSV</td>
<td>Herpes-simplex type-1 virus</td>
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<td>ICV</td>
<td>Intracerebroventricular</td>
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<tr>
<td>IFS</td>
<td>Inescapable foot shock</td>
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<tr>
<td>IL PFC</td>
<td>Infraalimbic prefrontal cortex</td>
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<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>ITC</td>
<td>Intercalated cells</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
</tr>
<tr>
<td>KPBS</td>
<td>Potassium phosphate buffered saline</td>
</tr>
<tr>
<td>LA</td>
<td>Lateral amygdala</td>
</tr>
<tr>
<td>LC</td>
<td>Locus Coeruleus</td>
</tr>
<tr>
<td>IITC</td>
<td>lateral intercalated cells</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
</tr>
<tr>
<td>mITC</td>
<td>medial intercalated cells</td>
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<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>NAcc</td>
<td>Nucleus accumbens</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione</td>
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<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitary</td>
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<tr>
<td>OF</td>
<td>Open field</td>
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<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
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<td>PBS</td>
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<td>PKG</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>PL PFC</td>
<td>Prelimbic prefrontal cortex</td>
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<td>PnC</td>
<td>Pontine reticular formation</td>
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<td>PP</td>
<td>Pancreatic polypeptide</td>
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<td>PPTg</td>
<td>Pedunculopontine tegmental nucleus</td>
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<td>PSS</td>
<td>Predator scent stress</td>
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<td>PTSD</td>
<td>Posttraumatic Stress Disorder</td>
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<td>PVN</td>
<td>Paraventricular nucleus</td>
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<td>PYY</td>
<td>Peptide YY</td>
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<td>rAAV</td>
<td>adeno-associated virus</td>
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<td>RAM</td>
<td>Radial arm maze</td>
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<td>Radioimmunoassay</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RT qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
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<tr>
<td>SCR</td>
<td>Skin conductance response</td>
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<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SNpc</td>
<td>Substania nigra pars compacta</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SPS</td>
<td>Single prolonged stress</td>
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<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
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<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
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<tr>
<td>vCA1</td>
<td>Ventral CA1</td>
</tr>
<tr>
<td>vmPFC</td>
<td>Ventromedial prefrontal cortex</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>Y1</td>
<td>Neuropeptide Y receptor 1</td>
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<tr>
<td>Y2</td>
<td>Neuropeptide Y receptor 2</td>
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<td>Y4</td>
<td>Neuropeptide Y receptor 3</td>
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<td>Y5</td>
<td>Neuropeptide Y receptor 5</td>
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<td>Y5</td>
<td>Neuropeptide Y receptor 6</td>
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CHAPTER 1

Post-Traumatic Stress Disorder (PTSD): Relevance of Neuropeptide Y (NPY)

I. Post-Traumatic Stress Disorder (PTSD)

Posttraumatic stress disorder (PTSD) is a function-impairing, trauma-evoked syndrome with an annual prevalence of about 4.7% and a life time prevalence of about 6.1% in the general adult population (based on the National Epidemiologic Survey on Alcohol and Related Conditions-III) (Goldstein et al., 2016). Higher prevalence rates of approximately 13% are observed in combat veterans (U.S. Department of Veterans Affairs, 2015).

The term PTSD was first described and listed as a clinical syndrome in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III) in 1980. However, the symptoms of the disorder had been long recognized and referred to by other names, such as “shellshock”, “war neurosis”, or “combat fatigue”. PTSD has generally been associated with combat trauma, and was first identified following World War I. During this time, it was believed that the symptoms of what was then called shellshock stemmed from exposure to the explosions of heavy artillery. Over time, clinicians began to understand that the condition was due to extreme psychological trauma experienced during combat. Following World War II, in 1952 the DSM-I was published which included a diagnosis called “gross stress reaction” that resembled what we now know as PTSD. The DSM-II, published in 1968, omitted any mention of gross stress reaction, as there were no ongoing wars with soldiers returning home with psychiatric symptoms. It was not until after the Vietnam War that the disorder was reconsidered when extensive psychiatric symptoms were observed in deployed US veterans returning home. Throughout the 1960s and 70s, extensive research was done on PTSD as a disorder caused by extreme trauma, including that experienced by civilians such as assault, mass tragedies, and accidents (Bloch, 1978; Parker, 1977; Sims et al., 1979; Wilson et al., 1965). The DSM-III was published in 1980 and defined PTSD as it is known today.

Since the DSM-III first defined PTSD, it has gone through various changes in symptom categories. With the recent publication of the DSM-V in 2013, PTSD has been re-classified from
an Anxiety Disorder to a Trauma and Stressor Related Disorder (see Table 1 for diagnostic criteria). The DSM-V requirements for PTSD are based on symptom criteria (A-E). Criteria A lists exposure to actual or threatened death, serious injury, or sexual violence that can be directly experienced, witnessed, or indirectly experienced through a close friend or family member. The other four symptom categories include intrusion symptoms (B), avoidance (C), negative alterations in cognition or mood (D), and alterations in arousal and reactivity (E). Intrusion symptoms include recurrent memories, traumatic nightmares, dissociative reactions (also known as flashbacks), and prolonged distress and physiologic reactivity following exposure to trauma reminders. Avoidance symptoms include avoidance of thoughts, feelings, and/or external reminders of the trauma. Cognitive and mood symptoms include an inability to recall key features of the traumatic event, persistent and distorted negative beliefs and expectations about oneself or the world, persistent distorted blame of self or others for causing the traumatic event or for resulting consequences, persistent negative trauma-related emotions, diminished interest in significant activities, feeling alienated, and persistent inability to experience positive emotions. Alterations in arousal and reactivity associated with PTSD include irritability, aggression, self-destructive or reckless behavior, hypervigilance, exaggerated startle response, problems with concentration, and sleep disturbances. As with other symptoms, these must have begun or worsened following the trauma. All symptom categories require at least one of the listed symptoms except for alterations in cognition or mood, which requires two. All symptoms must be present for at least one month, cause significant distress or functional impairment, and not be caused by medication, substance use, or other illness. A more concise explanation of PTSD symptom categories and individual symptoms can be found in Table 1.

Two new subtypes of PTSD have also been defined under the DSM-V: a Preschool subtype, which covers children under 6 years old, and a Dissociative subtype, which is defined as “PTSD with prominent dissociative symptoms (either experiences of feeling detached from
one’s own mind or body, or experiences in which the world seems unreal, dreamlike or distorted”.

II. PTSD Neurobiology

(1) Relevance of forebrain regions regulating fear, anxiety, and cognition

PTSD is a disorder of emotional learning. For this reason, brain systems that contribute to learning and memory as well as fear and emotions have been implicated. The amygdala is known to be the hub for fear memory processing in our brain. We know this based on multiple studies showing increased amygdala activity during fear (LaBar et al., 1998) as well as the lack of fear recognition in patients with amygdala lesions (Adolphs et al., 1994; Scott et al., 1997). For this reason, the amygdala has been one of the main brain areas studied in PTSD patients. Cognitive function and mood are also affected in PTSD. The PFC is well known for its role in cognitive function (Cabeza & Nyberg, 2000) and has been implicated in emotion regulation and depression (Rive et al., 2013). Multiple studies examining PTSD patients implicate either the prefrontal cortex (PFC), amygdala or both as being dysfunctional (Patel et al., 2012). Most of these studies use imaging techniques in order to assess size, functionality, and connectivity across various brain areas of PTSD patients versus healthy controls or those that have experienced trauma but do not meet the diagnosis requirements for PTSD (Hull, 2002; Lanius et al., 2006; Liberzon & Sripada, 2007). A recent meta-analysis of imaging studies in PTSD patients reveals that across different investigations the amygdala shows greater activity (hyperactivity) while the prefrontal cortex is hypoactive when compared to healthy controls (Patel, 2012). The hippocampus has also been implicated in PTSD due to its role in memory (Hattori et al., 2015; Nakamura & Sauvage, 2016) as well as fear (Lonsdorf et al., 2014; Nees & Pohlack, 2014). However, the evidence is not nearly as strong as the amygdala and PFC, due
to the lack of changes in function seen in the hippocampus. Specific studies related to PTSD are described below for each area.

(i) Amygdala

The amygdala is perhaps the most logical place to look for dysfunction in the brains of PTSD patients due to its role in fear memory and reactivity, the “fight-or-flight” response (Rasia-Filho et al., 2000). Hyperactivity in the amygdala has been a consistent finding in multiple neuroimaging studies in PTSD patients (Driessen et al., 2004; Hendler et al., 2003; Liberzon et al., 1999). PTSD patients have increased amygdala activity at resting state (S. B. J. Koch et al., 2016) and when reacting to emotional stimuli (van Rooij, Rademaker, et al., 2015), particularly negative emotional stimuli (Xiong et al., 2013). Increased amygdala activation to negative emotional stimuli prior to trauma exposure has been reported to have a strong association with posttraumatic symptoms later on (McLaughlin et al., 2014). Interestingly, when compared to a group of combat exposed veterans, a group of PTSD diagnosed veterans showed less prefrontal/parietal connectivity with the amygdala, and connectivity was inversely related to their avoidance scores (Simmons et al., 2011).

(ii) Prefrontal Cortex

Though it is less obvious than the amygdala, the prefrontal cortex has also been implicated in many studies as an area showing dysfunction in PTSD patients. This is not surprising given the PFC’s regulatory influence as a “break” system on the amygdala for emotional reactivity (Lee et al., 2012). MRI studies in humans have revealed that activation in the ventral mPFC, specifically Brodmann area 25 (which is most analogous to the infralimbic cortex (IL PFC) in rats) is related to reduced amygdala responses during extinction (Phelps et al., 2004), lending strength to the argument that the PFC exerts top down regulation over the amygdala. Hypoactivity within the PFC is perhaps one of the most consistent findings in PTSD patients (Bremner et al., 1999a;
Bremner et al., 1999b; Shin et al., 2004). In a study that compared male PTSD patients with trauma exposed control subjects, participants were given an fMRI scan while reacting to happy or fearful faces (Shin et al., 2005). The PTSD patients showed significantly diminished medial prefrontal cortex responses to fearful vs. happy faces compared to the control group. Another study investigated female PTSD patients who had experienced male-perpetrated interpersonal violence versus healthy females and found that the women with PTSD had lower ventromedial PFC activation to emotionally charged scenes of men and women interacting versus neutral interactions (Moser et al., 2015). The potential for the PFC to play a role in suppression of amygdala responses in humans coupled with direct evidence of hypoactivity in this region in PTSD patients, suggests that dysfunction in this area may contribute to the symptoms of PTSD.

(iii) Hippocampus

The hippocampus is an extremely important brain area for memory (Ranganath & Hsieh, 2016). Hippocampal lesions in rodents impair their ability to remember a sequence of movements in the radial arm maze (Chiba et al., 1994). In humans, the role for the hippocampus in memory is supported by increased hippocampal activity during learning of word triplets which correlates with increased memory for the order in which the words were learned (Tubridy & Davachi, 2011). The hippocampus has also been explicitly implicated in fear memory, particularly contextual fear memory, by multiple rodent (Christiansen et al., 2014; Gan et al., 2016; McHugh et al., 2013; Phillips & LeDoux, 1992; Thorsell et al., 2000; Tulogi et al., 2012) and human studies (de Voogd et al., 2016; Lissek et al., 2014; Onat & Büchel, 2015). When examining the hippocampus in PTSD patients, the most common finding is a reduction in volume. A recent meta-analysis of studies that looked at hippocampal volume in PTSD patients found that for all 36 studies examined a reduction in hippocampal volume of PTSD patients was reported when compared to either healthy or trauma exposed control patients (O'Doherty et al., 2015). Another study found that though hippocampal volume does not increase with treatment, but a smaller
hippocampal volume indicated an increased risk for persistence of PTSD (van Rooij et al., 2015). Due to the hippocampus’s obvious role in memory as well as fear, and the correlation between hippocampal volume reduction and PTSD, there is evidence to support a potential role for hippocampal dysfunction in PTSD patients.

(2) Stress regulatory Transmitter Systems implicated in PTSD: a brief update

Although the primary focus of this dissertation is Neuropeptide Y (NPY) (as described in sections III and onward), here we provide a brief update on other stress-regulatory systems that have been implicated in PTSD. NPY may work in concert with these transmitters/systems to impact PTSD-relevant behavior and physiology.

(i) Corticotropin-releasing Hormone (CRH)

CRH, a peptide hormone and neurotransmitter, is a prime component of the hypothalamic-pituitary adrenal axis (HPA) axis (Vale et al, 1981). CRH is mainly synthesized in the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus, but CRH mRNA is also highly expressed in the central amygdala (CeA), bed nucleus of the stria terminalis (BNST), and hippocampus (Wong et al, 1994). Within the HPA axis, CRH is released by the paraventricular nucleus of the hypothalamus (PVN) in response to physical or emotional stress and stimulates the pituitary to synthesize and release adrenocorticotropic hormone (ACTH), which then goes on to stimulate the release of cortisol and other stress hormones.

Elevated levels of CRH have been reported in the cerebrospinal fluid (CSF) of PTSD patients (Bremner et al., 1997). Overall, PTSD patients seem to have a hyperactive CRH system at a baseline, no stress condition (reviewed in Kasckow et al., 2001). Interestingly, in patients with combat-related PTSD exposure to a combat related video produced a drop in CSF CRH that was inversely related to self-reported anxiety levels (lower CRH, higher anxiety) (Geracioti et al., 2008). The authors postulate that this could be due to increased uptake of CSF...
CRH into brain tissue, increased CRH utilization, increased CRH degradation, or to an acute stress-related inhibition or suppression of CRH secretion.

Genetic studies have revealed an association of SNPs in the genes for CRH receptors 1 and 2 (CRHR1 and CRHR2) as well as CRH binding protein (CRHBP) with PTSD or protection from PTSD. In a sample of patients that had been admitted to the ICU, it was found that patients who were homozygous for the CRHBP rs10055255 T allele were less likely to have post-ICU PTSD (Davydow et al., 2014). In the same study, patients with aCRHR1rs1876831 C allele were more likely to present with post-ICU depression symptoms. Overall, there is strong evidence for a role of dysregulated CRH system in PTSD, likely associated with HPA dysfunction (discussed in the following section).

(ii) Hypothalamic-Pituitary-Adrenal Axis (HPA)

The HPA axis, comprised of the hypothalamus, the pituitary gland (a small structured located below the hypothalamus), and the adrenal glands (located on top of the kidneys), functions to provide the primary hormonal response to homeostatic challenge (Ulrich-Lai & Herman, 2009). Cortisol, released following HPA activation, is the major stress hormone in humans that activates both glucocorticoid (GR) and mineralocorticoid (MR) receptors. The HPA axis is self-regulated by negative feedback mechanisms, as cortisol activates forebrain receptors to limit the release of CRH from the PVN, and ACTH from the pituitary. These feedback mechanisms are crucial to the optimal functioning of the HPA.

Hypoactivity of the HPA axis response (reduced cortisol response to an emotional or pharmacological challenge) has been considered by some to be a hallmark of PTSD (reviewed in de Kloet et al., 2006 and Yehuda, 2002). Interestingly, a more suppressed cortisol curve following dexamethasone (a synthetic steroid that reduces ACTH release) challenge has been correlated with a greater reduction in PTSD symptoms following psychotherapy (Nijdam et al., 2015). Multiple studies have reported GR hypersensitivity in PTSD patients (Mcfarlane et al., 2010; Yehuda et al., 2008), a result of low cortisol levels in PTSD patients leading to enhanced
GR sensitivity. Increased GR sensitivity in PTSD has been associated with a single nucleotide polymorphism (SNP) in the GR gene Bcl, which correlates with PTSD symptoms (Castro-Vale et al., 2016). Lower levels of methylation of the NR3C1 promoter of the GR gene have been reported in PTSD patients in association with reduced cortisol in the dexamethasone suppression test (Yehuda et al., 2015). Female offspring of mothers that have been diagnosed with PTSD also show increased baseline cortisol level (Liu et al., 2016), suggesting that abnormalities in HPA function may show transgenerational effects.

(iii) Norepinephrine (NE)

NE, a monoamine stress regulatory transmitter is synthesized and released within the central nervous system (CNS) and the sympathetic branch of the autonomic nervous system (ANS). Within the CNS, NE is produced by the locus coeruleus (LC) within the pons. NE has been widely investigated in the regulation of attention, arousal, and the fight-or-flight response (reviewed in: Aston-Jones & Waterhouse, 2016; Berridge & Spencer, 2016; Chandler, 2016). In humans, NE increases heart rate and blood pressure, as well as elevates blood flow to the muscles, and triggers the release of glucose from the body’s energy stores (Tank & Wong, 2014). NE levels are low during sleep, increase with wakefulness, and reach peak concentrations following severe stress or trauma. NE has also been shown to be important for reconsolidation of both fear and drug-related memories (Otis et al., 2015). Direct administration of beta adrenergic receptor agonist propanol into the amygdala in rats enhances reconsolidation of fear and compromises extinction in an auditory fear conditioning paradigm (Dębiec et al., 2011), suggesting that NE may contribute to enhanced fear memory following a traumatic event.

Increased arousal, startle, and hypervigilance are all key symptoms of PTSD. Potential contributions of the noradrenergic system to psychophysiological reactivity in PTSD has been investigated by many studies (reviewed in Southwick et al., 1999). In a study comparing PTSD patients with and without co-morbid major depressive disorder (MDD) to those with MDD only or healthy controls, it was found that patients with PTSD alone had the highest unstimulated
plasma levels of NE (Yehuda et al., 1998). However, at least 3 other studies have found no difference in plasma NE levels between combat-related PTSD patients and healthy controls (Southwick, et al., 1995). In addition to plasma NE, lower alpha-2 adrenergic binding sites have been reported in PTSD patients (Perry et al., 1987). Men with combat-related PTSD had higher unstimulated CSF NE than healthy controls (Geracioti et al., 2001). In a cohort mainly consisting of African-American women, an association of rs2400707, a SNP in the promoter region of the beta-2 adrenergic receptor gene, along with childhood adversity, increased likelihood of adult PTSD symptoms (Liberzon et al., 2014). Efficacy of noradrenergic blockers such as propranolol (a beta-adrenergic blocker) as well as, clonidine and guanfacine (alpha-2 receptor agonists) have been tested in PTSD. A recent meta-analysis showed no effect of propranolol on PTSD symptom severity (Steenen et al., 2016) or during the acute phase after trauma for PTSD prevention (Argolo, 2015). In two trials comparing guanfacine with placebo, no improvement of PTSD symptoms was reported (Davis et al., 2008) and one trial showed an increase in side effects such as light-headedness and dry mouth. There have been no large, double blind, placebo controlled studies of alpha-2 receptor antagonist for PTSD, however improvements have been seen in clinical practice (Belkin & Schwartz, 2015). Thus, although the NE system appears to be associated with PTSD symptomology, especially the re-experiencing/intrusive cluster, therapeutic efficacy of this system is still debatable.

III. Neuropeptide Y

Experiencing or witnessing an intense traumatic event or events is a prerequisite to the development of PTSD. However, a significant variability is observed among individuals in
susceptibility to PTSD. The ability of the psyche to withstand severe, repeated traumas-or to rebound and recover from them-is the hallmark of psychological resistance or resiliency. Since only a fraction of trauma-exposed individuals develops PTSD, there has been considerable interest in the identification of biological factors that may confer resiliency versus susceptibility to PTSD. Mounting evidence suggests a potential role of stress regulatory transmitter NPY in the pathophysiology of PTSD. This dissertation investigates the contribution of forebrain NPY, specifically in the PFC and amygdala to PTSD-relevant behaviors simulated in rodent models. This section provides a translational perspective on the relevance of NPY in PTSD by collating preclinical, clinical and translational models.

(1) Neuropeptide Y (NPY) and NPY receptors: a brief overview

NPY is a 36 amino acid peptide that is widely distributed throughout the central and peripheral nervous system (Adrian et al., 1983; Allen et al., 1983). It is processed from a 97-amino acid precursor, pre-pro-NPY which is processed to the final peptide after catalytic action of prohormone converting enzymes and carboxypeptidase-like enzyme, and subsequently amidated to the biologically active form, NPY1-36 (Grouzmann & Brakch, 2005). The NPY family of peptides includes peptide YY (PYY) and pancreatic polypeptide (PP), two enteric peptides that share a hairpin-like structure called the PP-fold (Gehlert, 2004).

NPY messenger RNA (mRNA) mapping within the rodent brain identified the hypothalamic arcuate nucleus (ARC), the LC, the nucleus tractus solitari (NTS) and the septohippocampal nucleus as major sources of NPY synthesis (Kask et al., 2002). Investigation of NPY mRNA in the human brain tissue reveals abundance in the neocortex, polymorphic layer of the dentate gyrus (DG), basal ganglia and amygdala (Caberlotto et al., 2000). In conjunction with mRNA mapping, high NPY peptide expression is observed in cell bodies and fibers within the amygdala, nucleus accumbens (NAcc), various hypothalamic nuclei, cortex and hippocampus within the human brain (Adrian et al., 1983). In rodents, the highest expression
levels are seen in the hypothalamus, amygdala, cortex, hippocampus, NAcc, periaqueductal grey (PAG), dorsal raphe nucleus (DRN), the A1-A3 noradrenergic cell groups in the ventral medulla and the LC (Allen et al., 1983; Wahlestedt et al., 1989; Yamazoe et al., 1985).

The biological actions of NPY are mediated by G-protein coupled receptors. To date five different Y receptors (Y1, Y2, Y4, Y5, and Y6) have been cloned and characterized in mammals (Michel, 1991). While the Y1, Y2, Y4 and Y5 receptors are functional, the Y6 receptor is non-functional in several mammals, including humans, and is not present in rats (Bromée et al., 2006). NPY shows strong affinity for the Y1, Y2 and Y5 receptors, while PP is the preferential agonist at the Y4 receptor (Alexander et al., 2013).

Most NPY receptors associate with pertussis toxin-sensitive Gi/Go proteins that can trigger hyperpolarization of the cell (Michel, 1991; Michel et al., 1998). Intracellular signaling events include inhibition of calcium channels, activation of G protein-coupled inwardly-rectifying potassium (GIRK) channel activity or Ih inhibition via hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Acuna-Goycolea et al., 2005; Giesbrecht et al., 2010; Sun et al., 2001). NPY receptors reduce cyclic adenosine monophosphate (cAMP) via inhibition of adenylate cyclase and mobilize calcium through phospholipase C/phosphatidylinositol 3-kinase (PLC/PI3K) activity (Michel et al., 1998 and references therein). NPY receptors can also regulate gene transcription via activation of extracellular signal-regulated kinase (ERK) or CREB (cAMP response element–binding protein) signaling (Mullins et al., 2002; Sheriff et al., 2002). Table 2 illustrates Y receptor subtype ligand specificities and recruited signaling pathways (also refer to Brothers & Wahlestedt, 2010)

Y1 is the most abundant Y receptor in humans, with highest expression observed in the subcallosal gyrus and insular cortex, and moderate expression in the cingulate, frontal and temporal cortices (Caberlotto et al., 1997). Autoradiographic studies using a Y1-selective positron emission tomography ligand, Y1-973, demonstrate expression of the receptor in the human brain, including the DG, caudate-putamen, cortical regions, hypothalamus and thalamus (Hostetler et
Co-localized expression of NPY with receptor subtype Y2 is observed in the human cerebral cortex, hippocampus, amygdala, striatum and NAcc (Caberlotto et al., 2000a). Recent detailed immunochemical studies in the mouse brain support presynaptic localization of the Y2 receptor co-localized to NPY and GABAergic (gamma aminobutyric acid) terminals (Stanić et al., 2011). Evidence from site-selective ablation of the Y2 gene in the amygdala further supports a regulatory role of the Y2 receptor in presynaptic release of NPY as well as GABA (Tasan et al., 2010). Autoradiographic studies reveal significant species differences in the distribution of NPY receptor subtypes between mice, rats and primates (Dumont et al., 1998). $[^{125}I]-\text{Leu}^{31}, \text{Pro}^{34}-\text{PYY}$ (Y1-preferring)- binding sites are preferentially expressed in the cortex, hippocampus, hypothalamus and brainstem of the rat/mouse brain, whereas low overall expression was evident in the human brain except for the DG. Y2-preferring-$[^{125}I]-\text{PYY}^{3-36}$ sites are enriched in the hippocampus, cortex and septum in rodent brain, while a preferential distribution in the cortex was observed in human brain.

Most rodent behavioral models pertinent to PTSD have focused on Y1, Y2 and Y5 receptors given their high affinity for NPY and expression in areas regulating stress, anxiety and fear related behaviors. Contributions of each receptor subtype are discussed throughout the review where applicable. The differential, species-specific distribution of NPY receptors needs to be considered when extrapolating data from preclinical rodent models to humans. This becomes especially relevant for inferences drawn from rodent PTSD models where region-specific manipulation with selective NPY receptor agonists and antagonists may not apply to humans.

(2) Relevance of NPY in Post-Traumatic Stress Disorder

Accumulating evidence from preclinical and clinical studies conducted over the past decade strongly support a potential role of NPY in the pathophysiology of PTSD. Figure 1 illustrates a
temporal layout of major observations from preclinical, clinical and translational studies that have facilitated our understanding of a potential association of NPY with PTSD. The following sections discuss these observations lending support to the physiological relevance of NPY in PTSD.

(3) Preclinical Evidence: NPY regulates behavioral and physiological responses pertinent to PTSD

Experiencing or witnessing traumatic events can lead to PTSD, suggesting the relevance of biological systems that are recruited following trauma and stress exposure. NPY and NPY receptors in limbic and brainstem areas play an important role in the regulation of stress and anxiety (Reichmann & Holzer, 2015), fear (Tasan et al., 2016), learning and memory (Gøtzsche & Woldbye, 2015), and cardiovascular regulation (Zukowska-Grojec, 1995). Figure 2 illustrates NPY regulation of PTSD-relevant behavioral and physiological responses, highlighting brain areas that mediate these effects.

(i) NPY and stress

Collective evidence suggests that NPY regulates the HPA axis as well as the sympathetic nervous system (SNS), which are responsible for the response and adaptation to stress (Ulrich-Lai & Herman, 2009). The PVN of the hypothalamus is densely innervated by NPY terminals, some of which synapse on CRH neurons (Liposits et al., 1988). NPY administered by intracerebroventricular (ICV) injection or directly into the PVN elevates plasma ACTH and corticosterone in rats (Hanson & Dallman, 1995; Härfstrand et al., 1987). These effects may be mediated by Y1 and Y5 receptors, as central injections of the Y1 agonist [Leu31,Pro34] NPY and Y5 agonist [CPP1-7, NPY19-23 Ala31, Aib32, Gln34]hPP significantly increased plasma ACTH and corticosterone (Dimitrov et al., 2007; Kakui & Kitamura, 2007). NPY receptors have also been shown to regulate the HPA axis via extrahypothalamic sites. Conditional knockout mice lacking Y1 receptors in Ca2+/calmodulin-dependent kinase II (CaMKII)-expressing forebrain excitatory neurons (primarily hippocampus) have elevated corticosterone and increased NPY and CRH.
immunoreactivity in the PVN (Bertocchi et al., 2011). Thus, hippocampal Y1 receptors may play a role in inhibition of the HPA axis. However, studies on HPA activity in NPY deficient mice have yielded contradictory results. Chronic restraint stress-evoked HPA activation was increased in NPY-deficient mice (Baldock et al., 2014). Contrary to this, maternal separation stress-evoked HPA activation was attenuated in NPY-deficient mice (Schmidt et al., 2008). Timing, duration or modality of stress may be relevant in this regard. In a recent study, timing of stress exposure within the circadian cycle was found to be a relevant factor in NPY regulation of the HPA axis as well as stress-evoked anxiety and startle behavior. In this study, rats exposed to predator stress prior to the inactive phase demonstrated higher vulnerability to stress-evoked HPA activation, anxiety and startle responses compared to rats exposed prior to the active phase (Cohen et al., 2015a). Interestingly, NPY expression showed a higher magnitude of decrease in the inactive versus active cycle. Furthermore, NPY infusion into the PVN rescued stress induced neuroendocrine and behavioral responses associated with the diurnal cycle.

In addition to having direct effects on the HPA axis, the NPY system also operates as a physiological brake to counteract and regulate the activity of pro-stress transmitters such as NE and CRH that have been implicated in PTSD. Significantly elevated concentration of cerebrospinal fluid CRH and NE is observed in PTSD subjects (Baker et al., 1999; Geracioti et al., 2001). NPY and CRH are expressed in areas relevant to stress and emotional regulation, such as the amygdala, hypothalamus, and the BNST. Behavioral studies suggest that NPY exerts potent anxiolytic effects, whereas CRH is anxiogenic. Thus, it seems that a balance of these two peptides may exert an important influence on regulation of behavioral state. Direct injection of NPY in the basolateral nucleus of the amygdala (BLA) prior to a CRH agonist injection significantly blocks the development of avoidance behavior in the two floor choice test (Sajdyk et al., 2006; Wahlestedt et al., 1990) and prevents the CRH-induced reduction in social interaction time (Sajdyk et al., 2004). NPY and CRH have been reported to converge on GABA synapses in the CeA and the BNST, having opposing effects on projection neurons and downstream effector
regions impacting stress and anxiety-related behaviors and alcohol consumption (Gilpin et al., 2015; Kash & Winder, 2006; Pleil et al., 2015). Stimulatory effects of NPY on neuroendocrine responses via hypothalamic CRH may appear contradictory to inhibition of CRH responses by NPY in other brain regions, such as in the amygdala and the BNST. The differential effects of NPY on CRH transmission in these regions may be attributed to differential coupling to effector systems, as well as to localization of NPY receptors on inhibitory or excitatory circuits. Interaction of stress and appetite regulatory effects of NPY and CRH in the hypothalamus may also contribute to these differences.

NPY exists as a co-transmitter with NE in central and peripheral noradrenergic neurons (Hendry, 1993) where it regulates the release and activity of NE in sympathetic responses. Central and peripheral NPY regulate cardiovascular responses in association with NE, exerting significant decreases in blood pressure and heart rate when injected ICV and into the brainstem of rats (Morris & Pavia, 1997; Westfall et al., 2006), while exerting potent vasoconstrictor effects in the periphery (Wahlestedt et al., 1990). Mice exposed to six weeks of chronic stress manifest significant attenuation of NE and CRH release in the PVN by NPY neurons in the ARC, an effect that is absent in NPY deficient mice and restored following expression of NPY in NE neurons (Baldock et al., 2014). In addition to the hypothalamus, the LC appears to be another important site for NPY-NE recruitment following stress. Recent studies in the single prolonged stress (SPS) model of PTSD have shown normalization of SPS-evoked increases in HPA activity and elevated dopamine-β hydroxylase expression by intranasal (IN) NPY administration (Sabban et al., 2015; Serova et al., 2013).

Pharmacological and genetic studies in rodents support a pivotal role of NPY in promoting stress adaptation and coping. Transgenic rats overexpressing NPY show insensitivity to the normal anxiogenic-like effect of restraint stress in the elevated plus maze (EPM) (Thorsell et al., 2000). NPY administration into the BLA significantly reduced restraint stress-evoked anxiety in rats, an effect persistent over weeks (Sajdyk et al., 2008b).
(ii) NPY and anxiety

Central administration of NPY has potent anxiolytic effects in multiple models of anxiety-like behavior in rats and mice (Eaton et al., 2007; Kask et al., 2002; Reichmann & Holzer, 2015; Wu et al., 2011). Following ICV NPY treatment, anxiolytic effects were observed in the EPM (Broqua et al., 1995), the social interaction test (Sajdyk et al., 1999) and open field test (Sørensen et al., 2004), as well as models of learned suppression of behavior (e.g., non-operant punished drinking) (Britton et al., 1997). In spontaneously hypertensive rats, ICV NPY increased exploratory activity in the light-dark box (Pich et al., 1993). Site-specific studies have identified the amygdala (Sajdyk et al., 1999), PAG (Kask et al., 1998b), hippocampus, lateral septum (Trent & Menard, 2011) and LC (Kask et al., 1998a) as potential anatomical substrates responsible for anxiolytic effects of NPY (Kask et al., 2002). Pharmacological anti-anxiety effects of NPY agree with data from genetic studies. NPY deficiency is associated with an anxiogenic phenotype in mice (Bannon et al., 2000). Viral-mediated overexpression of NPY in the amygdala (Christiansen et al., 2014; Primeaux et al., 2005) or hippocampus (Christiansen et al., 2014; Lin et al., 2010) is anxiolytic in the EPM and open field test.

A large number of studies implicate the Y1 receptor in mediating attenuation of anxiety by NPY. Y1-preferring agonist [Leu^{31}, Pro^{34}]NPY produces NPY-like anxiolytic effects in the EPM (Broqua et al., 1995). Furthermore, Y1 receptor-selective antagonists, BIBO3304 and BIBP3226 block anxiolytic effects of NPY in the social interaction test (Sajdyk et al., 1999). Administration of BIBP3226 alone resulted in site-specific effects on anxiety-like behavior on the EPM (Kask et al., 1998b): anxiogenic behaviors were observed following injection into the PAG while no effects were observed in the amygdala, LC and PVN injected animals. Anxiolytic effects of NPY are absent in mice lacking Y1 receptors (Karlsson et al., 2008). Recently, conditional knockdown of Y1 receptor from excitatory forebrain neurons using CamKIIα promoter-CRE driver resulted in elevated anxiety in the EPM (Bertocchi et al., 2011). Anti-anxiety effects of NPY possibly result from altered excitability within circuits regulating anxiety. NPY via the Y1 receptor reduces
synaptic excitability in the BLA (Giesbrecht et al., 2010a; Molosh et al., 2013a). Y2 and Y5 receptors may also be recruited in NPY effects on anxiety, although the directionality of receptor actions appear to be region-specific. For example, Y2-agonism in the amygdala produces anxiogenic responses in the social interaction test (Sajdyk et al., 2002), whereas injection of Y2 agonists in the LC and lateral septum produces anxiolysis. Selective knockout of Y2 in GABAergic neurons in the CeA is anxiogenic in female but not male mice (McCall et al., 2013). On the contrary, CeA infusion of Y2 antagonist BIIE0246 reduced anxiety in the EPM in both naïve and alcohol-dependent rats (Kallupi et al., 2014). Global deletion of Y2 was also found to reduce anxiety in multiple tests of anxiety (Painsipp et al., 2008; Tschenett et al., 2003). Initial studies reported no effects of Y5 antagonist, CGP71683A on NPY evoked anxiolysis (Kask et al., 2001). However, subsequent studies using a different Y5 antagonist (Lu AA33810) demonstrated anxiolytic efficacy (Walker et al., 2009). ICV administration of Y5 agonist produced anxiolytic effects in the EPM and open field tests (Sørensen et al., 2004). Interestingly, conditional deletion of Y1 from Y5 expressing neurons results in increased anxiety-like behavior, suggesting a role of Y1-Y5 co-expressing neurons in regulating anxiety (Longo et al., 2015).

Overall, collective evidence from pharmacological and genetic studies suggest an anxiolytic role of NPY mediated primarily via Y1 and potentially Y5 receptors. Role of Y2 receptors in anxiety appears to be region-and circuit dependent, although there is some consensus on anxiogenic effects possibly via auto-receptor mediated reduction in synaptic NPY concentrations.

(iii) NPY, fear learning and memory

NPY is expressed in areas relevant to the processing of fear memories such as the amygdala, hippocampus and PFC, and modulates neuronal excitability in these areas. These findings have led to investigation of the role of NPY in the regulation of fear conditioning and extinction (see Tasan et al., 2016 for review). The first evidence for fear regulatory effects of NPY came from studies demonstrating inhibition of fear-potentiated startle by NPY and the Y1 agonist, Leu(31), Pro(34)]-NPY (Broqua et al., 1995). More recently, infusion of NPY into the BLA was
found to inhibit the expression of fear-potentiated startle as well enhance within session extinction, an effect mediated by Y1 receptor within the BLA (Gutman et al., 2008). In a contextual conditioning paradigm, robust reduction of conditioned fear and facilitated extinction was observed following ICV NPY administration, partially mediated via the Y1 receptor (Lach & de Lima, 2013). In contrast, attenuation of cue-conditioned fear by intra-amygdala NPY infusion was not blocked by Y1 antagonism (Fendt et al., 2009). Recent studies implicate Y2 receptors in the CeA in fear expression and extinction (Verma et al., 2015). ICV NPY significantly inhibited incubation of conditioned fear tested a month following fear acquisition, a model pertinent to delayed onset PTSD (Pickens et al., 2009). Assessment of fear behaviors has been studied in genetic models and parallel pharmacological observations. NPY knockout mice show accelerated acquisition, increased expression of conditioned fear as well as impaired extinction (Verma et al., 2012). These effects are less pronounced in Y1 knockouts and were not observed in Y2-deficient mice. Interestingly, Y1-Y2 deficient mice exhibit the robust fear phenotype observed in NPY knockouts suggesting a synergistic role of these receptors in fear regulation. Interestingly, deletion of Y2 receptors from GABAergic neurons led to increased fear acquisition in female but not male mice (McCall et al., 2013). Rats lacking dipeptidyl peptidase-4 (DPP-4), an enzyme that cleaves and inactivates NPY, exhibit improved fear extinction, likely due to elevated central NPY concentration (Canneva et al., 2015).

To date most studies have targeted the amygdala as a site for NPY regulation of fear conditioning. However, NPY and NPY receptors are abundant in cortical regions that regulate fear memory, specifically extinction and retrieval of extinguished fear. Reduced activation in the ventromedial PFC (vmPFC) has been reported in PTSD subjects in association with impaired retrieval of extinction (Milad et al., 2009). Recent studies by our group observed a significant impairment in the retrieval of extinction following infralimbic infusion of NPY in rats (Vollmer et al., 2016). Interestingly, carriers of NPY gene polymorphism rs16147 have elevated NPY expression in the medial PFC (mPFC) (Sommer et al., 2010b). Prefrontal NPY expression appears to be
stress-sensitive, as exposure of rats to chronic stress resulted in a significant increase in this area (McGuire et al., 2011). Elevated prefrontal NPY may compromise top-down regulation of regions such as the amygdala and result in impaired processing of conditioned fear, perhaps leading to increased vulnerability to PTSD.

The evidence suggests that NPY may regulate different aspects of fear learning and memory in a region-selective manner. While inhibitory effects of NPY on conditioned fear are well established, regulation of fear extinction and retrieval may be more complicated. These responses may arise from differential regulation of fear circuits in discrete brain areas by NPY and its receptors. Further investigation using region-circuit-cell specific approaches may be required to carefully tease out the role of NPY in fear memory regulation.

(iv) NPY and control of autonomic responses

NPY regulates autonomic responses in a complex manner via the brain and periphery. NPY injected in the brainstem of rats significantly decreases blood pressure and heart rate (Morris & Pavia, 1997). Transgenic rats overexpressing NPY have reduced blood pressure at baseline and during stress (Michalkiewicz et al., 2003). NPY-evoked hypotension is accompanied by reduced plasma catecholamine concentrations. Intrathecal injections of NPY have also been reported to have depressor effects (Chen et al., 1988). Other studies have reported no differences at baseline but increased sympathetic activity during stress in mice overexpressing NPY in noradrenergic neurons (Ruohonen et al., 2009). However, these effects were attributed to increased NPY release from the adrenal gland following sympathetic activation. In contrast to central effects, NPY released from postganglionic sympathetic neurons is reported to be a potent vasoconstrictor (Han et al., 1998). Chronic peripheral infusion of NPY leads to increased systolic blood pressure and cardiac dysfunction (Zhang et al., 2015). Overall, it appears that central NPY plays a major role in inhibition of the SNS, effects that oppose its peripheral actions. It would be important to consider
these disparate effects when therapeutic targeting options and the utility of peripheral NPY as a surrogate biomarker for central NPY is being considered.

(4) NPY and PTSD: supporting evidence from human studies

Two lines of investigation in humans support a potential contribution of NPY to PTSD pathophysiology: (a) clinical and gene association studies showing a role of NPY in stress, coping, resiliency, and (b) clinical studies in individuals with PTSD. Based on studies in rodent models, clinical studies were undertaken to assess effects on stress responses and stress coping in humans. An association of NPY with PTSD is supported by several clinical observations as described below:

(i) NPY, stress coping and resilience

Seminal studies in military survival training soldiers (Morgan et al., 2000) reported a negative association between plasma NPY levels with distress and poor performance scores following interrogation stress supporting a role of NPY in the behavioral effects of stress in humans. Individuals with higher NPY were “stress-hardy” and had better performance (in terms of interrogation behavior scores) identified by the Army training laboratory, while lower NPY was related to higher distress and symptoms of dissociation. Plasma NPY is primarily sympathetic and adrenomedullary in origin, and is linked to enhanced sympathetic activation under stressful conditions (see above). In another cohort of US Navy personnel, plasma NPY, cortisol and NE were significantly associated with survival school stress: greater levels of NPY release negatively correlated with psychological distress scores (Morgan et al., 2000).

(ii) NPY Genetics relevant to PTSD

Direct associations of NPY or NPY receptor gene polymorphisms with PTSD have not been reported to date. However, a large number of genetic studies on NPY haplotypes and gene polymorphisms have been associated with stress coping, affect, pain sensitivity and addiction, all of which are relevant to PTSD. NPY haplotypes predict low and high expression of NPY mRNA in post-mortem brain and lymphoblasts, as well as plasma NPY peptide levels (Zhou et al., 2008). Lower haplotype-driven
NPY expression was associated with higher emotion-induced activation of the amygdala, higher trait anxiety and diminished pain/stress-induced activations of the endogenous opioid neurotransmission in various brain regions (Zhou et al., 2008). While several NPY SNPs have been investigated in different human pathologies, rs16147, rs3037354 and -1002 T>G polymorphisms appear to be most relevant to stress-associated responses. In general, all these polymorphisms are associated with lower NPY expression, although tissue-specific differences may exist. NPY SNP rs16147 is reported to account for more than half variation in NPY expression in humans (Zhou et al., 2008). An interaction between NPY gene polymorphism rs16147 and early adversity was found to modulate stress responses in young adults (Witt et al., 2011). Contribution of rs16147 allele associates with stronger bilateral amygdala activation and slower response to treatment in anxious depression patients (Domschke et al., 2010). There appear to be regional differences in the effects of SNP rs16147 on NPY expression. In a study by Sommer et al, (Sommer et al., 2010) NPY SNP rs16147 resulted in higher prefrontal NPY expression in postmortem samples. In the same study, a separate epidemiological sample showed association of SNP rs16147 with negative affect in individuals exposed to high adversity. Differential NPY expression among brain regions may arise from variances in post-transcriptional processing or differences in epigenetic DNA modifications among brain regions (Hannon et al., 2015). Another NPY gene SNP, rs3037354 is reported to associate with elevated stress-evoked cardiovascular responses, higher plasma NPY and altered GR signaling (Zhang et al., 2012). Additionally, a loci in the NPY promoter (1002 T>G) results in lower NPY expression in the CSF and amygdala and higher arousal during stress and alcohol consumption in rhesus macaques (Lindell et al., 2010). Contrary to these findings, one study failed to replicate the contribution of NPY gene haplotypes to trait anxiety (neuroticism) (Cotton et al, 2009).

Interestingly, polymorphisms in NPY receptors have been associated with addictive behaviors. SNPs in the Y2 and Y5 receptor genes associate with alcohol dependence and comorbid cocaine dependence (Wetherill et al., 2008). NPY Y1 receptor polymorphism rs7687423 has been linked with methamphetamine dependence (Okahisa et al., 2009). Although direct evidence of NPY SNPs
association with PTSD has not been determined, a pilot prospective study reported greater susceptibility to PTSD in the absence of early intervention in a high risk group expressing combined genetic variants (including NPY rs16147) (Rothbaum et al., 2014). Collectively, these data suggest that genetic variation in NPY expression may promote inter-individual differences in stress and emotional responses to trauma that are relevant in determining PTSD susceptibility or resilience following trauma.

(iii) NPY in PTSD patients

Direct evidence for the relevance of NPY in PTSD pathophysiology comes from measurements of NPY peptide-like immunoreactivity in PTSD subjects. Table 3 compiles clinical studies on NPY measurements in PTSD subjects and other conditions that are often comorbid with PTSD.

Based on preclinical evidence our group tested the hypothesis that CSF NPY concentrations are reduced in PTSD patients. We reported significantly lower CSF NPY levels in Vietnam veterans with combat-related PTSD relative to healthy controls (Sah et al., 2009). In a follow up study we replicated reduced CSF NPY concentrations in Iraq/Afghanistan combat veterans with PTSD compared to a combat-exposed non-PTSD group (Sah et al., 2014), suggesting that low CSF NPY concentration is a pathophysiological feature of PTSD and not due to combat exposure per se. Moreover, NPY inversely correlated with intrusive symptom and diagnostic Clinician Administered PTSD scores (CAPS) but not with combat exposure scale (CES) and comorbid depression score (BDI), suggesting a possible association of reduced CSF NPY with PTSD symptomology. Of relevance to comorbidities related to PTSD, reduced CSF NPY has been observed in individuals with insomnia and substance dependence (Huang et al., 2015; Xu et al., 2012), while elevated CSF NPY was reported in individuals with impulsive aggression (Coccaro et al., 2012).

Plasma NPY concentrations in PTSD have been examined by other groups. Baseline plasma NPY levels were found to be reduced (Rasmusson et al., 2000) or unchanged (Morgan
et al., 2002) in PTSD patients as compared with healthy or trauma exposed non-PTSD subjects, respectively. Significantly higher plasma NPY levels were reported in individuals with past PTSD but currently recovered, suggesting a potential role of NPY in resilience (Yehuda et al., 2006). High coping and resilience showed positive correlation with NPY levels in these subjects.

Overall, CNS NPY concentrations appear to be associated with PTSD pathophysiology, although the exact contributions of NPY are unknown. Plasma NPY concentrations may be reflective of sympathetic drive, however, given recent studies showing a lack of correlation between CSF and plasma NPY pools (Baker et al., 2013), there is need for caution in extrapolating plasma to predict central brain NPY status. Future studies are required to compare these pools concurrently in PTSD subjects and comparing NPY changes at baseline versus stress conditions.

(5) NPY and PTSD: evidence from animal models of PTSD-like behavior

Following clinical observations of central NPY dysregulation in PTSD, numerous studies have investigated NPY regulation and intervention in rodent models simulating PTSD-relevant behaviors, with the objective of investigating potential mechanistic and pharmaco-therapeutic contributions of NPY. Previous studies have shown that exposure to acute and chronic stress regulates NPY expression in the brain, the magnitude and directionality being dependent on stressor modality, duration and brain area being examined (Reichmann & Holzer, 2015). In recent years, several animal models of PTSD have been proposed, primarily in rodents (Daskalakis et al., 2013; Goswami et al., 2013). Most models involve exposure of the animal to acute or chronic stressor/stressors to evoke a phenotype that simulates PTSD-like behaviors and physiology. These include increased fear expression, increased acoustic startle response (ASR), potentiated anxiety-like behavior, increased sympathetic responses, reduced social interaction, and altered sleeping behavior. In the following sections, changes in NPY and NPY receptor protein or mRNA expression as well as behavioral outcomes produced by NPY intervention are described for each model (collated in Table 4).
(i) Predator Scent Stress (PSS) Models

**Regulation of NPY in PSS model**

This model entails exposure to the scent of a natural predator to mice or rats. Exposure of rats to soiled cat litter for 10 min produced reduced NPY-like immunoreactivity in a wide range of brain areas including posterior cortex, amygdala, hippocampus, and the PAG at 7 days post-PSS exposure (Cohen et al., 2012). Reduced NPY is specific to the subset of animals expressing increased anxiety-like behaviors on the EPM and an increased ASR. In a recent study, NPY expression was associated with differential sensitivity to predator exposure across the circadian cycle (Cohen et al., 2015). Basal expression of NPY specifically in the hypothalamic PVN and ARC was significantly lower at the onset of the light phase but not at the dark phase onset (Cohen et al., 2015). Interestingly, rats exposed to PSS at the onset of light cycle (but not the dark cycle) also manifested HPA abnormalities, increased ASR and reduced open arm time in the EPM.

**NPY Intervention in PSS model:**

Based on PSS-evoked reductions in hippocampal NPY, animals were administered 5 or 10µg bilaterally directly into the dorsal hippocampus, 1 hr. post-PSS exposure (Cohen et al., 2012). A significant attenuation of PSS-induced increases in ASR amplitude, contextual fear and anxiety-like behavior was observed in NPY treated animals. Additionally, NPY treatment resulted in elevated NPY and YR1 immunoreactivity in the DG and brain-derived neurotrophic factor (BDNF) immunoreactivity in the dentate and CA3 regions. In a more recent study, NPY intervention in the PVN normalized PSS-induced increases in ASR, elevated anxiety-like behavior and HPA response (Cohen et al., 2015).

(ii) Predator Exposure

Similar to PSS, exposure of rodents to a natural predator constitutes an intense stressor relevant to threat simulation for PTSD, although animals are never in direct contact with the predator (Daskalakis et al., 2013; Goswami et al., 2013; Stam, 2007). Typical predators include cats (for rats) or rats (for mice) (Adamec et al., 2004; Cohen & Zohar, 2004). Predator stress-
evoked behavioral manifestations such as HPA hyperactivity, anxiety-like behavior on the EPM and light-dark box and exaggerated ASR are measured at delayed intervals ranging from 7d to 30d (Daskalakis et al., 2013; Stam, 2007). A recent study investigated NPY alterations in a predator exposure paradigm using captured field mice (Varman & Rajan, 2015). Predator-exposed field mice reared under standard housing conditions exhibited increased anxiety-like behavior and fear. However, field mice reared under enriched conditions were resistant to predator-evoked behavioral impairments. Interestingly, a significant elevation of NPY was observed in the amygdala of enrichment-exposed mice that did not express predator stress effects. Furthermore, expression of the Y1 receptor in the amygdala is significantly increased and that of the Y2 receptor significantly decreased in the enriched housing group. This is consistent with the anxiolytic/anti-stress versus anxiogenic role of the Y1 and Y2 receptors, respectively.

(iii) Single Prolonged Stress (SPS) model

*Regulation of NPY in SPS model*

The SPS model involves exposure of animals to a 2 hr. restraint stress, a 20 min forced swim (24°C) and ether exposure, consecutively. This model is reproducible in multiple laboratories, making it a popular PTSD animal model (Lee et al., 2014; Liberzon et al., 1997; Nedelcovych et al., 2015; Sabban et al., 2015; Serova et al., 2013; Yamamoto et al., 2009). SPS evokes a delayed onset HPA dysregulation, marked by enhanced negative feedback inhibition of ACTH responses to corticosterone injection or re-stress in the form of restraint (Liberzon et al., 1997). Attenuated NPY mRNA expression in the amygdala (Nedelcovych et al., 2015), and reduced NPY peptide immunoreactivity in the PVN (Lee et al., 2014) are observed at 7 and 9 days post-SPS, respectively. Significant SPS-evoked decrease in NPY Y2 receptor mRNA in the LC has also been reported (Sabban et al., 2015). In contrast, one study observed a significant increase in NPY immunoreactive fibers and terminals specific to the BLA (not CeA) at 7d post SPS exposure in rats (Cui et al., 2008). In this study double immunostaining by fluorescence and electron
microscopy revealed that NPY immunoreactive terminals were closely associated with CaMKII (a marker for pyramidal neurons)-positive neurons in the BLA, which were also immunopositive for GR and MR. Since no other endpoints were investigated, it is not evident how these SPS-evoked morphological changes relate to PTSD-relevant physiology and behavior. In a predator stress model, decreased dendritic length and increased branch points within the BLA were observed only in a subset of stress-exposed animals that exhibited low anxiety scores on the EPM (Mitra et al., 2009). Collectively, SPS evoked changes in NPY expression appear to be dependent on brain region. Assessment of behavioral outcomes in conjunction with morphological measurements may be necessary for correct interpretation of stress-evoked NPY changes.

NPY Intervention in SPS model

IN administration of NPY in the SPS model has proved to be effective at reducing behavioral and neurochemical changes induced by SPS (Serova et al., 2013; Serova et al., 2014). One study compared IN NPY treatment in SPS treated animals either 30 min before or immediately after SPS exposure (Serova et al., 2013). IN administration of NPY prior to SPS improved PTSD- and depression-related behaviors (reduced ASR, increased open arm time on the EPM, and reduced immobility in forced swim test (FST) tested at 7d post-SPS. IN NPY blocked the expression of many SPS-evoked neurochemical changes, including elevated mRNA expression of CRH, FKBP5 and GR in the hypothalamus and GR protein in the hippocampus (Laukova et al., 2014).

In a separate study, IN NPY increased tyrosine hydroxylase (TH) protein in the LC and increased CRH receptor mRNA and protein in the CeA (Sabban et al., 2015). In addition to its efficacy as a preventive treatment for PTSD-like behaviors, the same team tested whether IN NPY can reverse SPS-evoked behavioral impairments in a follow up study. When IN NPY was administered after the 7d recovery period (when behavioral impairments are evident), significant changes were observed on several PTSD-relevant behavioral endpoints (attenuated ASR, reduced anxiety and depression-like behaviors). These data suggest that IN administration of NPY may reverse
behavioral impairments triggered by the traumatic stress of SPS, suggesting a possible therapeutic potential for treatment of PTSD (Serova et al., 2014).

(iv) Inescapable foot shock (IFS) stress model

Regulation of NPY in IFS model

Foot shocks encompassing a wide range of intensity, duration and frequency have been commonly used as “traumatic stimuli” in PTSD animal models (Daskalakis et al., 2013; Goswami et al., 2013). Using a light dark box, where animals received 10 foot shocks upon crossover to the dark compartment, enhanced context–associated fear and generalized anxiety is observed post recovery (Hendriksen et al., 2012). While one can argue whether the concept of ‘trauma’ can be ascribed to rats and mice, like other models this method produces late emerging and lasting changes in behavior reminiscent of PTSD symptoms. A significant reduction of NPY Y1 receptor mRNA (but not NPY) was observed in the amygdala of shock-exposed animals at 22 d post-IFS exposure (Hendriksen et al., 2012). Interestingly, this reduction is rescued if animals are re-exposed to the shock context (without shocks) for 8 consecutive days (extinction) suggesting recruitment of amygdala Y1 receptor in PTSD-relevant behaviors in this model.

NPY Intervention in the IFS model

Based on observations of Y1 receptor recruitment in the IFS model (see above) the Y1 receptor agonist [Leu31,Pro34]-NPY was administered into the BLA 20 minutes prior to behavioral testing at 21d post exposure to IFS. Infusion of Y1 agonist normalized the enhanced sensitivity to stress observed in IFS exposed animals when tested in the sudden silence test (SOS), a test where animals are placed in the open field with 85dB background noise, which is suddenly turned off after 5 min. Y1 agonist treatment decreased freezing and increased locomotion compared to vehicle treated animals. Freezing behavior was positively correlated with the distance of the cannula tip from the BLA, highlighting the specificity of the BLA to this effect (Hendriksen et al., 2012).

(v) Chronic Variable Stress (CVS) Model
Models using chronic stress exposure are relevant to PTSD, given evidence that the cumulative effects of chronic trauma such as combat stress may contribute to the disorder. We used chronic variable stress (CVS), composed of multiple, single episode events occurring in an unpredictable fashion, to simulate prolonged stress (McGuire et al., 2010; McGuire et al., 2011). CVS consists of a 7d, twice-daily exposure to physical and psychological stressors administered in an unpredictable manner. In a recent study, a final inescapable foot shock stressor within the CVS was included to simulate a stressful event that could later tested for post-recovery fear recall (Schmeltzer et al., 2015). The 7-day CVS exposure produces a pronounced deficit in HPA axis stress reactivity that emerges 4 days after cessation and persists for at least 30 days (Ostrander et al., 2006), consistent with reduced HPA drive seen in PTSD (Yehuda, 2001). The 1-week CVS procedure including the final inescapable foot shock also resulted in a blunted acoustic startle response. Although this may seem counterintuitive, others have reported blunted startle responses following chronic stress (Bijlsma et al., 2010; Conti & Printz, 2003). Interestingly, blunted startle reactivity was reported in individuals with cumulative traumatization-evoked PTSD in comparison with patients with discrete traumatic exposure (Lang & McTeague, 2011; McTeague et al., 2010).

Region-specific alteration in NPY is observed within key stress and fear-regulatory brain areas. At the 7d post CVS recovery period, animals exposed to CVS showed differential regulation of NPY with a significant reduction in the amygdala but a significant increase in the PFC (McGuire et al., 2011). These changes temporally associate with exaggerated fear memory and arousal behaviors observed in these animals, including enhanced freezing in response to a reminder stimulus (McGuire et al., 2010). NPY alterations are consistent with maladaptive emotional responses due to the nature of NPY signaling within these respective regions, as well as the reciprocal connectivity between the amygdala and the PFC. In a later study, inclusion of inescapable foot shocks within the CVS resulted in a significant reduction of hippocampal NPY at the 7d post recovery time point (Schmeltzer et al., 2015). Thus, adaptive changes in NPY may be
dependent on stressor modality and intensity within the CVS paradigm. Studies on NPY intervention in chronic traumatization models of PTSD has not been performed to date.

The CVS model was utilized in some of the studies that will be described in subsequent chapters. We believe this model to be relevant to chronic traumatization PTSD, due to the similar changes in HPA reactivity and startle response we have seen. Importantly, this model shows changes in two of the primary brain areas that show dysfunction in PTSD patients, the PFC and the amygdala. These two areas show changes in NPY protein levels at 7 days following CVS (McGuire et al., 2011), the same time point that exaggerated fear memory and arousal (McGuire et al., 2010) as well as changes in ASR (Schmeltzer et al., 2015). Due to this models relevance to PTSD and the lack of studies of NPY intervention in chronic traumatization models, we have chosen to utilize this model in studies comparing acute and chronic trauma as well as our long term over expression study.

In conclusion, the weight of evidence suggests that significant region-selective changes in NPY expression may correlate temporally with PTSD- relevant physiological and behavioral responses in most paradigms, although directionality of effects may not resonate among studies. It is evident from most models that the hippocampus, amygdala and the hypothalamus may be key areas where stress-sensitive effects of the NPY system are observed. Another area is the PFC, where NPY regulation of extinction retrieval has been reported and may be relevant to PTSD (Vollmer et al., 2016). More investigation on prefrontal NPY in rodent PTSD models is warranted. Importantly, intracranial NPY intervention into the hippocampus and hypothalamus or via IN administration appears to be effective in reducing enhanced anxiety-like behavior and ASR, normalizing HPA reactivity and fear related behaviors evoked by traumatic stress exposure. Collectively, these preclinical studies support the potential attractiveness of NPY intervention in PTSD prevention and treatment. An important consideration for extrapolating from rodent models to humans is the differential NPY receptor expression between the two species (see section 3, Part 1).
(6) Relevance of NPY in PTSD comorbid conditions

The evidence presented in preceding sections strongly supports an association of NPY with PTSD pathophysiology. In addition to the regulation of stress, anxiety and fear by NPY, as well as clinical studies on NPY in PTSD, it is important to note that NPY regulates other physiological responses that may contribute to comorbidities that are often associated with PTSD. These include the regulation of metabolism, nociception, addiction and depression-associated behaviors. Comorbidity of PTSD with physical illnesses and other psychiatric disorders has been identified. While it is beyond the scope of the current article to discuss each of these areas in detail, excellent reviews are referenced where applicable.

PTSD patients are at risk for developing obesity and type 2 diabetes (Farr et al., 2015; Vaccarino et al., 2014). Furthermore, PTSD has also been associated with obesity, particularly in women subjects (Kubzansky et al., 2014; Perkonigg et al., 2009). Given the interactions between stress and metabolism and a role of NPY in both appetite regulation (Stanley et al., 1986) and stress (see above), it is possible that NPY dysregulation in PTSD impacts metabolic outcomes. A recent review discusses this topic and the impact of NPY systems on body weight and metabolism in association with stress (Rasmusson et al., 2010).

Presence of chronic pain is observed in approximately 10-50% of PTSD patients suggestive of high comorbidity between the two conditions (Sharp & Harvey, 2001). PTSD-pain comorbidity is sustained by the perception of pain as a recurring trauma reminder increasing the individual’s level of perceived pain, emotional distress and disability. A close association with ongoing anxiety as an exacerbating factor for pain has also been proposed (Brennstuhl et al., 2015). Anti-nociceptive actions of NPY have been widely studied in several rodent models of pain (reviewed in Brumovsky et al., 2007). Pain/stress- induced activation of the endogenous opioid system, as measured by positron emission tomography (PET), was higher in individuals with greater NPY expression (Zhou et al., 2008); this finding is consistent with better suppression...
of pain and stressful stimuli in this population. NPY in the BNST inhibits the affective component of pain by opposing CRH induced conditioned place aversion (Ide et al., 2013). Reduced NPY tone in PTSD subjects may contribute to pain hypersensitivity, although these associations remain to be investigated.

Substance abuse comorbidity is present in nearly half of all PTSD patients (Breslau et al., 2003). A three-to five-fold higher incidence has been reported compared to healthy controls. Among individuals diagnosed with PTSD, the highest comorbidity is observed for alcohol dependence, followed by other substances such as opioids and cannabinoids, although stimulants like cocaine are also abused (Jacobsen et al., 2001). Conversely, approximately 25% of individuals with some addiction may suffer from some form of PTSD (Driessen et al., 2008).

Regulation of the neurobiological response to addictive substances by NPY, including drugs of abuse (such as psychostimulants), nicotine and alcohol (including alcohol consumption, dependence, and withdrawal) is well established (Gilpin & Roberto, 2012; Gonçalves et al., 2015; Thorsell, 2007). The current consensus is that NPY deficiency is associated with increased sensitivity to cocaine, elevated alcohol consumption and nicotine dependence (Bhaskar et al., 2013; Wetherill et al., 2008). Interestingly, the rs16147 polymorphism described above for stress and anxiety is also associated with increased risk for tobacco dependence (Mutschler et al., 2012). It is possible that maladaptive NPY function contributes to both PTSD and addiction physiology in individuals expressing comorbid symptoms.

Preclinical and clinical studies over the past several years support an association of NPY in the pathophysiology of depression (extensively reviewed in Eaton et al., 2007; Kormos & Gaszner, 2013; Morales-Medina et al., 2010; Wu et al., 2011). Significant comorbidity is observed between depression and PTSD, with about 36-43 % of traumatized subjects expressing comorbid PTSD and depressive symptomology (Campbell et al., 2007; Stein & Kennedy, 2001). As for PTSD, stress has a pivotal role in the pathophysiology of depression. Stress has been identified as a risk factor for both major depressive disorder and PTSD, and together with genetic
contributions results in psychopathological outcomes (Smoller, 2015). Measurements of CSF NPY in subjects with major depression have yielded inconsistent results. Initial reports showed significantly reduced CSF NPY levels in major depression (Heilig, 2004; Nikisch et al., 2005; Widerlöv et al., 1988). In contrast, other studies report no differences or significantly higher CSF NPY concentration in depressive subjects (Martinez et al., 2012; Soleimani et al., 2015). These inconsistencies may be explained by different subgroups within depression that may express higher NPY either as an adaptive response or due to genetic history (Soleimani et al., 2015). Recently, IN NPY was found to reverse depressive-like behavior evoked by SPS, a rodent model of PTSD (Serova et al., 2014). Studies on NPY association with comorbid PTSD and depression are lacking. In a recent study, we reported an inverse correlation of CSF NPY with PTSD diagnostic CAPS scores, but no associations with Beck Depression Inventory (BDI) scores in PTSD subjects with depression symptoms (Sah et al., 2014).

Evidence reported in the preceding paragraphs suggests that various PTSD comorbid conditions may also be associated with NPY dysregulation reported in PTSD. However, association studies in patient populations as well as the development of animal models simulating comorbid phenomena are required to understand specific contributions of NPY to PTSD and comorbidities.

(IV) PTSD-relevant Rodent Behavioral Paradigms

Rodent behavioral tasks have proven useful in simulating behaviors that may be representative of PTSD-relevant outcomes. For example, the phenomena of acquisition and extinction of fear behavior in animals may relate to certain aspects of PTSD pathophysiology such as the persistent memories of traumatic events. Enhanced startle amplitude may be representative of increased physiological reactivity representative of hyperarousal. PTSD-comorbid conditions such as persistent anxiety and depression maybe modeled by behavioral tests such as the open-field and forced swim paradigms, respectively. Cognitive problems may be modeled by the
prefrontal-dependent Delayed-Win Shift task. Although rodent models fall short of accurately simulating any psychiatric syndrome as a whole, collective investigation of several behavioral read-outs that represent various symptom clusters is a more realistic approach for preclinical studies. The following section provides a brief background on the behavioral paradigms that were used in the current dissertation for investigating contributions of NPY to PTSD-like behaviors.

(1) Auditory Fear Conditioning

As described in the previous sections, PTSD is a disorder resulting from persistent memories of trauma; therefore, fear regulation is central to our understanding of PTSD. The basis for PTSD is an exposure to intense trauma that is later experienced in the absence of the originating traumatic event. Auditory cued fear conditioning has been the gold standard for studying fear behavior and circuitry since John Watson and Rosalie Rayner’s experiments with baby Albert B. in the 1920’s. In these studies, they paired a loud aversive sound (the unconditioned stimulus, US) with the presence of a white rat, training baby Albert to fear the rat (the conditioned stimulus, CS) which he was originally unafraid of (Watson & Rayner, 1920). However, the origins of fear conditioning are in Pavlovian or, classical conditioning, a simple form of associative learning (Pavlov, 1927). Pavlovian conditioning is simply the act of associative learning when a CS predicts an US. Auditory fear conditioning takes this a step further in that it aims to teach a certain auditory stimulus, or tone, signifies an aversive event, in our case a foot shock. This paradigm is referred to as “fear conditioning” because the initially innocuous CS, after being paired with the US, elicits behavior similar to innate fear responses in the animal. These responses include freezing (lack of movement, except for movement required for respiration) (Curzon et al, 2009), which is the most popular and easily scored read-out for fear in rodents, as well as heart rate, blood pressure, respiration rate, and ultrasonic vocalizations (Hott
et al., 2016; Mileykovskiy & Morales, 2011; Prus et al., 2014). Fear conditioning paradigms usually include 3 phases: training, extinction, and extinction retrieval.

At the initiation of the training session, animals are exposed to habituation tones in the absence of foot shocks to signal that the tone is an innocuous stimulus. The habituation phase also allows the animals to habituate to the novel environment. Animals learn the association between the tone and shock quickly, usually after only 1 or 2 CS-US pairings. Training is also referred to as acquisition because animals are acquiring a new memory of this CS-US association. This form of associative learning is dependent on the amygdala (Wilensky et al., 1999), and the changes in lateral amygdala (LA) responses to the CS mirror those seen in hippocampal long-term potentiation (LTP), a form of neural plasticity thought to underlie memory formation (Rogan et al., 1997).

Following training, a period of time passes (anywhere from 12 hrs. to up to 1 month). After this time passes, animals are again presented with the tone, but in the absence of the foot shock. Animals will display visible signs of conditioned fear, such as freezing. Other measurements can also be made to determine conditioned fear, as mentioned previously. Over time, within (or between) sessions where animals are re-exposed to tone in the absence of shock, animals will begin to reduce their fear-like responses. This is known as extinction. Extinction has been defined as “the decrement in conditioned fear responses that occurs with repeated presentation of a conditioned fear stimulus that is unreinforced” (Milad & Quirk, 2012). Extinction of fear has direct relevance to PTSD, where deficits in extinction learning have been reported (Blechert et al., 2007; Milad et al., 2008; Wessa & Flor, 2007). The process of extinction is similar procedurally to exposure therapy often used to treat anxiety disorders and PTSD, where patients are exposed to feared objects, contexts, or memories in a safe space in order to overcome the associated fear. In rodent models, extinction involves the repeated presentation of the CS without the US, and can be done in 1 day or in multiple sessions over
multiple days. Many studies have demonstrated that the process of extinction does not replace the initial memory of CS-US association, but creates a competing CS-no US association memory (Bouton, 2004; Quirk & Mueller, 2008). Extinction itself can be further divided in acquisition, consolidation, and retrieval, with different process and brain areas playing roles in each. We know that during acquisition of extinction active learning is occurring, rather than entirely inhibitory processes, due to studies demonstrating extinction dependency on protein synthesis (Flood et al., 1977) as well as N-methyl-D-aspartate (NMDA) receptors in the BLA (Falls et al., 1992), similar to conditioning (training).

After some period of time following extinction, animals are tested for extinction retrieval, also referred to as “recall” or “extinction recall”. Extinction retrieval involves exposing the animal again to CS (tone-only) trials in order to evaluate the level of recall the animal has for the extinction session. The vmPFC, particularly the IL, have been shown to be important for the retrieval of extinction memory, as lesions of this area result in normal extinction behavior but reduced recall of extinction the following day (increased freezing to tone) (Quirk et al., 2000). In addition, single cell recordings from projection neurons in the IL of rats have shown that their firing signaled tones during the extinction retrieval phase, and the strength of their firing was inversely correlated with freezing during extinction retrieval (Milad & Quirk, 2002). Lower levels of freezing to the CS indicate good retrieval of the extinction memory. High levels of freezing indicate poor retrieval of or lack of formation of extinction memory, possibly due to pathological process interfering with formation, consolidation, or retrieval of extinction memory. Extinction has been shown to be context specific, though context can refer to all the sensory cues present in the physical location, as well as the internal state of the animal. This means that if the training context (A) is different than the extinction context (B), then the extinction memory can only be expressed if the tone is presented in context B. The same is true for retrieval of extinction if tested in context B, when training and extinction have occurred in context A, it is likely the
animal will revert to conditioned fear responses when exposed to the CS during extinction retrieval (Bouton, 1993).

Following extinction retrieval, other behaviors can be observed, such as the recovery of responses to the CS following exposure to a context different from that of extinction (renewal). This process has been shown to be dysregulated in PTSD patients (Garfinkel et al., 2014). Both PTSD patients and combat exposed controls were trained to associate a light with a shock in context A and extinguished the following day in context B. The next day subjects were exposed to the light in context B to test extinction recall (impaired in PTSD patients, as previously noted in this section) and context A to test fear renewal. Fear renewal, as measured by skin conductance response (SCR), was significantly lower in PTSD patients, showing a reduced capacity to use contextual information to modulate fear. Reinstatement of the CS-US association can be accomplished by exposing animals to a single US trial, followed by CS-only trials in order to measure the strength of the reinstated CS-US association memory. Reinstatement is not usually looked at in PTSD patients, as this would be counter-productive to their recovery. However, at least one study has shown that performing extinction immediately following re-activation of a fear memory (as opposed to 6 hours later or with no re-activation) in healthy subjects is able to prevent reinstatement of fear up to a year later (Schiller et al., 2010). Another test than can be run following extinction and extinction retrieval is reconditioning. Reconditioning involves exposing the animals to one paired CS-US reminder trial followed by multiple CS-only trials in order to test the strength of the extinction memory versus the strength of the initial training memory. One study conditioned Gulf War Veterans with and without PTSD to associate a contextual cue with both a wrist shock and loud auditory stimulus (Grillon & Morgan, 1999). Conditioning and extinction were performed during session 1, and 4-5 days later the entire procedure was repeated. PTSD patients showed increased startle response to the second conditioning session (reconditioning) (measured by electrodes place below the eye to measure muscle twitch) indicating, as suggested by the authors, that PTSD may be more
associated with deficits in learning safety cues than in deficits in the activation of mechanisms of fear inhibition.

(2) Acoustic Startle Response (ASR)

Hyperarousal or hypervigilance is a hallmark symptom of PTSD. Many studies have shown an increased startle response using skin conductance (Grasso & Simons, 2012), eye blink response (Butler et al, 1990) and heart rate (Orr et al., 1997) in PTSD patients. In order to simulate hyperarousal in rodents an acoustic startle task was used (Matar et al., 2006; Rajbhandari et al., 2015; Serova et al., 2013; Servatius et al., 1995). The ASR in rodents is the reaction physical response that occurs in the animal in response to an unexpected loud noise. In rodents, the response includes contraction of the facial and skeletal muscles, closing of the eyes, and stiffening of the neck and body (Koch, 1999). ASR can be influenced by situational factors such as a history of stress (Adamec et al., 1999; Schmeltzer et al., 2015), pairing with a conditioned fear response (fear potentiated startle) (Davis & Astrachan, 1978), or giving a pre-pulse sound before the startle stimulus (pre-pulse inhibition) (Ison et al., 1973). The version of the task used in studies conducted in this dissertation involves placing the animal in a plastic tube enclosure large enough for the animal to turn around, which is placed on a piezoelectric transducer pad that measures force, all within a soundproof enclosure. When the startle stimuli (a short, loud burst of noise) is presented, the animal reacts by a sudden contraction of muscles, the force of which can be measured by the piezoelectric transducer pad below it. This method allows for assessment of acoustic startle without invasive techniques, and has been used to measure hyperarousal in multiple animal models of PTSD.

(3) Open Field OF

A high level of underlying comorbid anxiety is a hallmark of PTSD, and has been associated with longer duration of illness (reviewed in Brady et al., 2000). Though PTSD is no longer classified as an anxiety disorder under the DSM-V, many of the symptoms are related to,
cause high levels of anxiety in patients. To simulate anxiety-like behavior in rodents, the OF test was conducted in our investigations. The open field is a commonly used behavioral paradigm to measure anxiety-like behavior in rodents (reviewed in Buccafusco, 2009) and has been used since the 1930’s (Hall, 1934). This test involves placing an animal in a large square arena, that is considerably larger than their normal home cage environment. The arena has tall sides to avoid escape and is divided into defined areas such as the center and border spaces. Rodents are placed in the open field arena and movement throughout the field is videotaped and tracked for anywhere from 3 to 10 min. The preference and time spent in specific areas as well as total distance travelled is measured. This test was originally based on behavior of animals in the wild that tend to become very still when a predator is nearby in order to decrease their chances of being detected. Exposure to an open environment without shelter also tends to increase autonomic nervous system activity in rodents, which leads to increased defecation (Denenberg, 1969). These behaviors (freezing and defecation) as well as open-field behavior correlation with anxiety, were confirmed by measuring animals distance travelled and fecal boli during a 3min. exposure to an open field (Levine et al., 1967). It was found that animals who moved around more and had less fecal boli in the field have lower levels of plasma corticosterone. Due to novelty of the environment of the open field, information about exploratory behavior can also be assessed from this test. The open field’s ability to measure both anxiety-like and exploratory behaviors makes it extremely useful when evaluating animal models of stress and trauma.

(4) Forced Swim Test (FST)

As previously mentioned, PTSD is highly comorbid with depression. In a recent meta-analysis of PTSD comorbidity studies, all 16 studies that considered depression found a significant comorbidity of PTSD and depression between 23 and 71% (Greene et al, 2016). Modeling depression-like behavior in rodents is difficult because the hallmarks of depression are changes in mood that are inaccessible in animals. Therefore, we must use models that display
phenotypes of depression that we can measure such as behavioral despair, or lack of escape behavior in an undesirable context. The FST was used to evaluate behavioral despair in our animals. It is a measure of “learned helplessness” in which animals who have previously experienced stress are less likely to display appropriate escape seeking behaviors and become immobile and passive sooner than others. This test is often mistakenly referred to as a test or model of depression. However, since the behavior that the animal displays during the test is a direct result of the test itself (being placed in an inescapable water tank) and is not present in the animal’s everyday undisturbed behavior, it is best thought of as a test of the antidepressant effects of a treatment and not a model of depression itself (Buccafusco, 2009). The FST was first described in 1977 by Porsolt in a paper in which it was demonstrated that immobility in the forced swim test was reduced by agents known to be effective treatments for depression (iprindole, mianserin and viloxazine) that had not previously shown effects with other animal models for testing antidepressant effects (Porsolt, 1977). Porsolt then went on to show that the forced swim test was able to distinguish between antidepressants (decrease in immobility), psychostimulants (decrease in immobility and increase in general activity), and tranquilizers (increase in immobility and general activity). He also showed an antidepressant effect of enriched environment (group housing in a maze-like environment with varied stimuli such as paper towel rolls, running wheels, and wooden blocks) (Porsolt, 1978). These data suggest that the FST is a very sensitive measure of the antidepressant effects of behavioral and pharmacological manipulations in rodents.

(5) Delayed Win-Shift Task (DWS)

Hypoactivity in the prefrontal cortex has been implicated in PTSD (Moser et al., 2015; Shin et al., 2005). PTSD patients have also shown deficits in prefrontal-dependent cognitive tasks such as attention, planning, and problem solving (Hayes et al., 2012). In order to test prefrontal dependent functioning, we have used the DWS task as a test of working and delayed
memory. This is based off the early work of Olton and Samuelson in which they introduced a delay in the radial arm maze task and noticed that rats did not change the number of correct responses (Olton & Samuelson, 1976). In the more current version, rats are tested for working memory by only visiting each baited arm once. After a delay, the previously unbaited arms are baited and all arms are open. Rats are tested for delayed memory by measuring the number of times they visit the previously baited arms (between phase errors) and the number of times they re-visit the currently baited arms after retrieving the bait (within phase errors) (McKlveen et al., 2016). This test has been shown by multiple studies to be heavily dependent on prefrontal cortex functioning and performance is impaired when the prefrontal cortex is damaged or inactivated (Di Pietro et al, 2004; Livingston-Thomas et al., 2015).

(V) Objectives of Dissertation

On the basis of aggregate data from the literature discussed in the preceding sections, we hypothesize that NPY will modulate conditioned fear and extinction memory via the prefrontal cortex and the amygdala. Additionally, NPY in these areas will regulate anxiety, startle, depression-associated behaviors, and cognitive function. This overarching hypothesis will be tested in the following set of experiments:

Aim 1

Study 1 will determine the role of NPY in the infralimbic prefrontal cortex in regulation of fear extinction and retrieval, depression-like behavior, cognitive function and HPA axis response.

Study 2 will characterize NPY projections to the infralimbic prefrontal cortex to understand circuits influencing prefrontal NPY function.
Aim 2

**Study 1** will determine the regulation of conditioned fear, extinction and retrieval by NPY administered into the BLA.

**Study 2** will determine the regulation of fear-associated behavior, anxiety-like and startle behaviors in rats following lentiviral NPY overexpression in the amygdala.

Aim 3

This Aim will determine PTSD relevant fear memory and acoustic startle behaviors in rats exposed to chronic versus acute stress and the regulation of NPY in the PFC, amygdala and hippocampus.
<table>
<thead>
<tr>
<th>Symptom Category</th>
<th># required for PTSD diagnosis</th>
<th>Symptoms</th>
</tr>
</thead>
</table>
| **Exposure to actual or threatened death, serious injury, or sexual violence** | 1 | Directly experiencing the traumatic event  
Witnessing, in person, the event as it occurs to others  
Learning that the traumatic event occurred to a close family member or friend (must be violent or accidental)  
Experiencing repeated or extreme exposure to aversive details of the traumatic event  
(Does not include electronic media, TV, movies, or pictures unless work related) |
| **Intrusion Symptoms** | 1 | Recurrent, involuntary, and intrusive distressing memories of the traumatic event  
Recurrent distressing dreams in which the content and/or effect of the dream are related to the traumatic event  
Dissociative reactions (flashbacks) in which the individual feels or acts like the traumatic event is recurring  
Intense or prolonged psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event  
Marked physiological reactions to internal or external cues that resemble an aspect of the traumatic event |
| **Persistent avoidance of stimuli associated with the traumatic event** | 1 | Avoidance of or efforts to avoid distressing memories, thoughts, or feelings about or closely associated to the traumatic event  
Avoidance of or efforts to avoid external reminders (people, places, conversations, activities, objects, situations) that arouse distressing memories, thoughts, or feelings about or closely associated with the traumatic event. |
| **Negative alterations in cognition or mood** | 2 | Inability to remember an important aspect of the traumatic event (not due to factors such as head injury, alcohol, or drugs)  
Persistent or exaggerated negative beliefs or expectations about oneself, others, or the world  
Persistent, distorted cognitions about the cause or consequences of the traumatic event that lead the individual to blame themselves or others  
Persistent negative emotional state (fear, horror, anger, guilt, or shame)  
Markedly diminished interest or participation in significant activities  
Feelings of detachment or estrangement from others  
Persistent inability to experience positive emotions (happiness, satisfaction, or loving feelings) |
| **Marked alterations in arousal and reactivity** | 2 | Irritable behavior and angry outbursts (with little or no provocation) typically expressed as verbal or physical aggression toward people or objects  
Reckless or self-destructive behavior  
Hypervigilance  
Exaggerated startle response  
Problems with concentration  
Sleep disturbance (difficulty falling or staying asleep, restless sleep) |

*Must be associated with the traumatic event (Category A) and begin or worsen following the traumatic event. Must last more than a month, cause clinically significant distress or impairment in social, occupational, or other areas of functioning, and not be associated with the physiological effects of a substance (medication, alcohol, etc.) or another medical condition.

Adapted from: American Psychiatric Association (2013). Diagnostic and statistical manual of mental disorders (5th ed.)
Table 2
Y receptor subtype ligand specificities and recruited signaling pathways

<table>
<thead>
<tr>
<th>Y receptor subtype</th>
<th>Ligand preference</th>
<th>Agonists [antagonists]</th>
<th>Cell Signaling ** (coupled to Gi/Go/Gq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>NPY&gt;PYY&gt;PP</td>
<td>[Leu^{31}, Pro^{34}]NPY (Y1/Y5)<em>, F7P^{34} NPY [BIBO3304,BIBP3226,1229U91, GR231118</em>, J-104870, J-115814, BW1911U90, BMS193885]</td>
<td>cAMP/PKA/PLC-IP3-Ca^{2+} GIRK channel ( I_h ) current (HCN channel)</td>
</tr>
<tr>
<td>Y2</td>
<td>NPY=PYY&gt;PP</td>
<td>PYY/NPY(3–36), PYY/NPY(13–36), [ahx^{5–24}]NP, [BIIE0246, JNJ-3102008, JNJ-5207787, SF-11]</td>
<td>cAMP-PKA PLC-IP3-Ca^{2+} PI3K-ERK Ca^{2+} channel</td>
</tr>
<tr>
<td>Y4</td>
<td>PP&gt;PYY=NPY</td>
<td>PP, BVD-74D, GR231118, Obinepitide [UR-AK49]</td>
<td>PLC-IP3-Ca^{2+} cAMP-PKA</td>
</tr>
</tbody>
</table>

*Y1 preferring; ** Refer to text for references
Table 3
Cerebrospinal fluid (CSF) and plasma NPY alterations in PTSD and PTSD-comorbid conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Pool</th>
<th>Alteration in NPY</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTSD</td>
<td>CSF</td>
<td>↓ NPY vs healthy volunteers</td>
<td>Sah et al, 2009</td>
</tr>
<tr>
<td>PTSD</td>
<td>CSF</td>
<td>↓ CSF NPY vs combat-no PTSD group</td>
<td>Sah et al, 2014</td>
</tr>
<tr>
<td>PTSD</td>
<td>Plasma</td>
<td>↓ baseline NPY</td>
<td>Rasmusson et al, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ yohimbine-stimulated NPY vs healthy volunteers</td>
<td></td>
</tr>
<tr>
<td>PTSD</td>
<td>Plasma</td>
<td>↓ baseline NPY in combat-PTSD and combat-no PTSD group vs healthy volunteers</td>
<td>Morgan et al, 2003</td>
</tr>
<tr>
<td>PTSD</td>
<td>Plasma</td>
<td>↑ baseline NPY in combat cohort with past PTSD vs combat cohort with no PTSD</td>
<td>Yehuda et al, 2006</td>
</tr>
<tr>
<td>Primary Insomnia</td>
<td>Plasma</td>
<td>↓ NPY (morning levels)</td>
<td>Huang et al, 2015</td>
</tr>
<tr>
<td>Substance Dependence</td>
<td>Plasma</td>
<td>↓ Stress-induced increases in plasma NPY (compared to healthy controls)</td>
<td>Xu et al, 2012</td>
</tr>
<tr>
<td>Impulsive Aggression</td>
<td>CSF</td>
<td>↑ CSF NPY-like immunoreactivity correlates with ↑ Impulsive aggression scores</td>
<td>Coccaro et al, 2012</td>
</tr>
</tbody>
</table>

↓ = decrease; ↑ = increase
<table>
<thead>
<tr>
<th>Model (Species)</th>
<th>NPY Expression</th>
<th>NPY Intervention</th>
<th>Intervention Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predator Scent Stress (PSS) (rat)</td>
<td>(7d post-PSS) ↓ NPY peptide ELISA (posterior cortex, amygdala, hippocampus, PAG) ↓ NPY (immunohistochem) (hippocampal CA1, dentate))</td>
<td>NPY dorsal hippocampus (1 hr. post-PSS)</td>
<td>Compared to aCSF (7d post-PSS) ↑ EPM Open arm time ↓ startle amplitude ↑ NPY and Y1R in DG ↑ BDNF in CA3 and DG ↓ contextual freezing</td>
<td>Cohen et al, 2012</td>
</tr>
<tr>
<td></td>
<td>(8d post-PSS) ↓ PSS (prior to light cycle) ↓ NPY (immunohistochem) (Arcuate nucleus, CA1, dentate, BLA) ↓ PSS (prior to dark cycle) ↓ NPY (immunohistochem) (CA1, dentate, BLA)</td>
<td>NPY PVN (30 min. prior to PSS)</td>
<td>Compared to aCSF (7d post-PSS) ↑ EPM open arm time ↓ startle amplitude ↑ PVN NPY, Y1r immunoreactivity</td>
<td>Cohen et al, 2014</td>
</tr>
<tr>
<td>Single Prolonged Stress (SPS) (rat)</td>
<td>(7d post-SPS) ↑ NPY peptide (amygdala) ↑ NPY (immunohistochem) (BLA)</td>
<td>ND</td>
<td>ND</td>
<td>Cui et al, 2008</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NPY Intranasal (30 min prior to SPS)</td>
<td></td>
<td>Compared to vehicle (7d post SPS) ↓ immobility in FST ↑ EPM open arm entries and risk assessment ↓ acoustic startle amplitude ↓ TH mRNA</td>
<td>Serova et al 2013</td>
</tr>
<tr>
<td>Study</td>
<td>NPY Intranasal (9d post-SPS)</td>
<td>Immobility in FST</td>
<td>NPY Immunoreactivity (PVN)</td>
<td>CRH mRNA (ventral hippocampus, hypothalamus)</td>
</tr>
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<tr>
<td>Lee et al 2014</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Compared to vehicle:</td>
</tr>
<tr>
<td>Laukova et al, 2014</td>
<td>Intranasal NPY (immediately post-SPS)</td>
<td>ND</td>
<td>Intranasal NPY (immediately post-SPS)</td>
<td>Compared to vehicle:</td>
</tr>
<tr>
<td>Serova et al, 2014</td>
<td>Intranasal NPY (7d post-SPS)</td>
<td>ND</td>
<td>Intranasal NPY (7d post-SPS)</td>
<td>Compared to vehicle:</td>
</tr>
<tr>
<td>Nedelcovych et al 2015</td>
<td>(7d post-SPS)</td>
<td>ND</td>
<td>(7d post-SPS)</td>
<td>NPY mRNA (amygdala)</td>
</tr>
<tr>
<td>Condition</td>
<td>Time Post-Stress</td>
<td>Intranasal NPY (immed. post-SPS)</td>
<td>Compared to Vehicle (7d post SPS):</td>
<td>References</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------------</td>
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</tr>
<tr>
<td>Inescapable Foot Shock (IFS) (rat)</td>
<td>22 days post-IFS</td>
<td>ND</td>
<td>TH protein and immunoreactivity (LC)</td>
<td>Hendriksen et al, 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y1R mRNA (amygdala)</td>
<td>CRH R1 mRNA (LC)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>CRH peptide (CeA)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Y2 receptor mRNA (LC)</td>
<td></td>
</tr>
<tr>
<td>Predator Exposure (PE) (mice)</td>
<td>7h post PE</td>
<td>ND</td>
<td>ND</td>
<td>Varman et al, 2015</td>
</tr>
<tr>
<td>Chronic Variable Stress (CVS) (rat)</td>
<td>7 days post-CVS</td>
<td>ND</td>
<td>ND</td>
<td>McGuire et al, 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPY peptide (amygdala)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPY peptide (PFC)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Chronic Variable Stress-Shock (CVS-S) (rat)</td>
<td>9 days post CVS-S</td>
<td>ND</td>
<td>ND</td>
<td>Schmeltzer et al, 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPY peptide (Hippocampus)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Inescapable Foot Shock (IFS) (rat)</td>
<td>22 days post-IFS</td>
<td>ND</td>
<td>ND</td>
<td>Hendriksen et al, 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPY-Y1 agonist [Leu31, Pro34]</td>
<td>(21d post-IFS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLA (21d post-IFS)</td>
<td>locomotion in SOS test</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>freezing in SOS test</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(SOS = stress of sudden silence)</td>
<td></td>
</tr>
<tr>
<td>Predator Exposure (PE) (mice)</td>
<td>7h post PE</td>
<td>ND</td>
<td>ND</td>
<td>Varman et al, 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y1 mRNA (amygdala)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y2 mRNA (amygdala)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ↓ = decrease; ↑ = increase; ND = not determined
Figure 1: Temporal trajectory of scientific observations from preclinical, clinical and translational animal models supporting an association of NPY with PTSD pathophysiology. Note the transition from preclinical studies to clinical and PTSD animal model studies forming a cycle of “bench” to “bedside” to “bench”. Preclinical observations supporting NPY regulation of stress, anxiety, autonomic regulation, memory and pain led to human trials on NPY and stress resiliency and coping. These observations were followed by CSF studies reporting NPY dysregulation in PTSD patients and genetic studies linking NPY polymorphisms with stress coping and resiliency. Ongoing preclinical studies on NPY and fear suggested potential links to PTSD symptomology. Clinical findings on NPY deficiency in PTSD prompted translational studies in animal models testing therapeutic potential of NPY intervention. Future studies are required on regional and circuit-based approaches to understand molecular mechanisms linking NPY to PTSD physiology. Preclinical observations on regional disparity in NPY function further necessitate the investigation of regional NPY status in postmortem brain tissue in PTSD. Human intervention studies will require an improved understanding of blood brain barrier permeability, CSF dynamics and novel NPY analogs for efficacious NPY targeting to the brain.
Figure 2: Potential sites for the regulation of behavioral and physiological responses relevant to PTSD and other comorbid conditions by NPY. Selection of target sites and responses are based on preclinical studies described in the text. (↑ = increase; ↓ = decrease).

mPFC = medial prefrontal cortex, LS = lateral septum, NAcc = nucleus accumbens, Hipp = hippocampus, BNST = bed nucleus of the stria terminalis, PVN = paraventricular nucleus of the hypothalamus, Amyg. = amygdala, Arc = arcuate nucleus of the hypothalamus, PAG = periaqueductal grey, RMg = nucleus raphe magnus, LC = locus coeruleus, NTS = nucleus of the solitary tract.
CHAPTER 2

Study 1:

Functional Role of Neuropeptide Y in the Infralimbic Prefrontal Cortex

Abstract

Background: Neuropeptide Y (NPY), a 36 aa peptide, regulates stress and emotional behaviors. Carriers of NPY gene polymorphism rs16147 have been reported to have elevated prefrontal NPY expression. In the current study, we examined functional attributes of NPY in the infralimbic (IL) cortex, an area that regulates fear memories and is reported to be hypoactive in PTSD. Preclinical and clinical studies support an association of NPY with trauma-evoked syndromes such as posttraumatic stress disorder (PTSD), although the exact contribution of NPY is not clear.

Methods: Bilateral cannulas were implanted in the infralimbic prefrontal cortex (IL-PFC) and the following 3 experiments were performed on separate groups of animals: (1) NPY dose response; (2) NPY effects on consolidation; (3) Y1 antagonist (n=12/group). Patch-clamp recordings from IL projection neurons were done in the presence of NPY to determine effects of NPY on neuronal excitability within this area. IL NPY effects on other mPFC regulated outcomes, such as the hypothalamic–pituitary–adrenal (HPA) response, forced swim test (FST) behavior, and working memory (delayed spatial win-shift task), were also measured.

Results: Infusion of NPY into the IL cortex in rats significantly impaired fear extinction memory without affecting conditioned fear expression or acquisition of extinction. Neuroendocrine stress response, depression-like behavior, and working memory performance were not affected by NPY infusion into the IL. The NPY Y1 receptor antagonist BIBO3304 completely abolished NPY effects on fear extinction retrieval. Y1 receptor expression was localized on CaMKII-positive pyramidal projection neurons and GAD67-positive interneurons in the IL. Patch-clamp recordings revealed increased inhibitory synaptic transmission onto IL projection neurons in the presence of NPY.

Conclusions: NPY dampens excitability of IL projection neurons and impairs retrieval of extinction memory by inhibiting consolidation of extinction. Of relevance to PTSD, elevation of
prefrontal NPY attributable to the genetic polymorphism rs16147 may contribute to IL hypoactivity, resulting in impaired extinction memory and susceptibility to the disorder.
Introduction

Neuropeptide Y (NPY), a 36 aa peptide, is widely expressed in the central nervous system (CNS) (Adrian et al., 1983; Allen et al., 1983) and regulates physiological and behavioral responses such as stress and anxiety, fear, learning and memory, control of blood pressure, and sympathetic activity (Eaton et al., 2007; Heilig & Widerlöv, 1995). NPY has gained attention as a stress resiliency transmitter associated with posttraumatic stress disorder (PTSD) (Sah & Geracioti, 2013; Wu et al., 2011), although the exact contributions of the peptide to PTSD pathophysiology are still unclear. Regulation of fear memories is pertinent to PTSD pathophysiology (Parsons & Ressler, 2013). An inability to suppress or extinguish fear memories may sustain pathologically high levels of fear in patients with PTSD years after the trauma (Jovanovic et al., 2010; Milad et al., 2009). Increasing evidence supports a primary role of the infralimbic subdivision (IL) of the medial prefrontal cortex [mPFC; ventromedial PFC (vmPFC in humans)] in fear extinction and retrieval (Milad & Quirk, 2002). Importantly, impaired retrieval of extinction observed in PTSD subjects is associated with reduced activation in the vmPFC (Milad et al., 2009). NPY and NPY receptors, particularly the Y1 receptor (Y1R), are abundantly expressed in cortical areas (Dumont et al., 1996) where they may impact synaptic excitability and function. Interestingly, higher prefrontal NPY expression is observed in individuals carrying the rs16147 T>C polymorphism in the NPY gene, which is associated with negative affect in high adversity (Sommer et al., 2010). In a rodent model of PTSD-like behaviors, we have shown a persistent elevation in prefrontal NPY mRNA and peptide that correlate temporally with exaggerated fear rein-statement and extinction deficits (McGuire et al., 2010; McGuire et al., 2011). NPY has potent inhibitory effects on neuronal excitability and has been shown to have strong anticonvulsant actions in neocortical circuits (Bacci et al., 2002). Studies to date on NPY regulation of fear have primarily focused on the amygdala (Gutman et al., 2008; Verma et al., 2015). However, no information exists currently on the functional contributions of NPY in the mPFC. Given the relevance of the IL cortex in PTSD
pathophysiology, we investigated the effects of IL NPY infusion on fear extinction and retrieval, a PTSD-relevant outcome. We also performed patch-clamp recordings from IL projection neurons in the presence of NPY to determine effects of NPY on neuronal excitability within this area. Other mPFC regulated outcomes, such as the hypothalamic–pituitary–adrenal (HPA) response, forced swim test (FST) behavior, and working memory (delayed win-shift task, DWS), were also measured (Butts et al., 2011; Radley et al., 2006; Wulsin et al., 2010). HPA dysfunction, depression, and cognitive deficits are often comorbid with PTSD (McNally, 2006; Vythilingam et al., 2010). Moreover, recent work suggests a role of the IL cortex in HPA regulation and depression-like behaviors (McKlveen et al., 2013). Immunohistochemical studies were also performed to localize NPY and NPY Y1 receptor expression in IL neurons.

Materials and Methods

Subjects
A total of 210 male Sprague Dawley rats (275–300 g; Harlan Laboratories) were used in the study. Rats were housed in polyethylene cages and maintained on a 12 h light/dark schedule with ad libitum access to standard laboratory rat chow and water. Experiments were conducted during the light phase. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Separate cohorts of animals were used for each experiment as follows: (1) NPY dose response (48; 12 per group); (2) NPY consolidation (24; 12 per group); (3) Y1 antagonist experiment (n = 48; 12 per group); (4) HPA (n = 24; 12 per group); (5) FST (n = 24; 12 per group); (6) delayed spatial win-shift (DWS; n = 24; 12 per group); (7) electrophysiology (n = 13); and (8) immunohistochemistry (n = 5). For behavioral experiments, only data from IL hits was used for statistical analyses (see section for details).

Surgery
Rats were anesthetized with ketamine/xylazine (87/13 mg/kg) and placed in the stereotaxic apparatus. The skin was retracted and the cranium was exposed and scraped. Twenty-six-gauge bilateral guide cannulas (Plastics One) were implanted over the IL cortex using the following coordinates: ±2.7 mm anteroposterior, ±0.6 mm mediolateral, and -5.1 mm dorsoventral from bregma (Paxinos, 2005). Cannulas were secured to the skull with stainless steel screws and dental cement. Rats were handled daily for 2 weeks, and cannulas were manipulated to habituate animals to the injection procedure. Obturators were removed and injector tips were placed into the guide cannulas. Injector tips had a 3.1 mm projection beyond the 2 mm guide cannulas. After infusion, injectors were left in place for 1 min to allow for the drug to diffuse.

**Drugs and infusions**

Timing of NPY administration at pre-extinction and post-extinction training were selected based on recent studies showing the necessity of the IL cortex during extinction training and extinction consolidation but not during retrieval of extinction (Do-Monte et al., 2015). NPY (Bachem) or artificial CSF (aCSF) was administered 30 min before extinction training on Day 2 (Fig. 1C) or immediately after extinction training (Fig. 1E). For dose response, we tested NPY at 3, 10, and 30 pmol/0.2 µl per side. The 10 pmol NPY dose was selected for all other experiments because it has also been used previously for NPY effects on fear potentiated startle extinction and stress via the basolateral amygdala (BLA) (Gutman et al., 2008; Sajdyk et al., 2008). The NPY Y1R antagonist BIBO3304 (N-[(1 R)-1-[[[[4[(aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4 [[(aminoiminomethyl)amino]butyl]-a-phenyl-benzeneacetamide ditrifluoroacetate (BIBO); Tocris Bioscience, R&D Systems] was infused at 200 pmol/0.2 µl per side, 10 min before NPY for antagonism experiments. This BIBO dose was selected because it effectively blocked NPY-facilitated extinction of fear-potentiated startle via BLA infusion (Gutman et al., 2008). For assessment of drug spread, we injected a few rats with fluorescent NPY (FAM-NPY; Phoenix Peptides), and fluorescence was assessed in hits verified by tracts.
within the IL coordinates (Fig. 1B). NPY was administered 30 min before restraint stress (HPA), FST exposure, or retrieval testing phase of the DWS task.

**Behavioral procedures**

**Fear conditioning.** Auditory fear conditioning, extinction, and retrieval of extinction were performed in operant chambers housed in sound-attenuated isolation cabinets (CleverSys) throughout all phases of the experiment. The floor of the chambers consisted of stainless steel grid bars that delivered scrambled electric footshocks. Between each run, the grid, floor trays, and chamber walls were wiped with 10% ethanol and allowed to dry completely. The auditory conditioning–extinction–retrieval procedure was adapted from a previous study (Sierra-Mercado et al., 2011). Briefly, on Day 1, all animals underwent training that consisted of five habituation tones [70 dB, 2 kHz, 30 s with a 3 min intertrial interval (ITI)] that were followed by seven conditioning tones that co-terminated with footshocks (0.6 mA, 0.5 s). On Day 2, animals were returned to the chambers for extinction training that consisted of exposure to 15 tones (70 dB, 2 kHz, 30 s, 3 min ITI) in the absence of footshocks. On Day 3, rats received 10 tones (70 dB, 2 kHz, 30 s with a 3 min ITI) to measure retrieval of extinguished fear. Conditioned fear responses to tone were measured as percentage freezing during 30 s. Freezing, defined as the absence of all movements except respiration, was measured from saved videos by observers blinded to treatment. Reported data analyses were performed on hand-scored data.

**FST.** The behavioral apparatus was a Plexiglas cylinder 45 cm high and 20 cm in diameter filled with 31 cm of water (30 –33°C). Rats were placed in the cylinder for 10 min, and the session was videotaped. An observer blind to the treatment conditions performed the scoring. The behavior was scored every 5 s, and the total counts for immobility behavior, which is defined as the rat not making any active movements or floating in the water without struggling, were assessed for the session, summed for each animal, and averaged within each treatment group.
**DWS procedure.** The DWS task was adapted from previous studies with modifications (Butts et al., 2011; Floresco et al., 1997) and performed on an eight-arm radial arm maze (RAM). After recovery from surgery, rats were food restricted to 80% of their ad libitum feeding for 1 week before training for the DWS procedure. The procedure involved three phases: habituation, training trials, and retrieval testing performance after aCSF and NPY. After recovery from cannula surgery, animals were habituated to the maze environment for 2 d (Days 1–2). Subsequent training trials were given once daily (Days 3–14). Each trial consisted of an acquisition phase and retrieval phase, separated by a delay period. During the acquisition phase, a set of four arms were chosen and blocked. Food pellets (Research Diets) were placed in the food cups of the four remaining open arms. Each rat was given 5 min to retrieve the pellets from the four open arms and then was returned to its home cage for the delay period. During the retrieval phase of each daily trial, all arms were opened, but only the arms that were blocked previously contained food. Rats were allowed a maximum of 5 min during the retrieval phase. Errors during the retrieval phase were scored as any entry into a nonbaited arm and were divided into two error subtypes: (1) “across-phase” errors that were defined as an entry into an arm that had been entered previously during the acquisition phase; and (2) “within-phase” errors defined as any reentry into an arm that had been entered in the retrieval phase. The initial delay period was 5 min until the rats reached criterion (one error or less during retrieval phase) for at least 2 consecutive days. The delay period was then increased to 30 min for another 2 days to confirm criterion of one or less error. On the final day of testing, aCSF or NPY was administered before the delay period, and errors during retrieval phase were recorded.

**HPA axis response to acute stressor**
Peripheral blood was collected from the tail vein at 15, 30, 60, and 120 min from initiation of restraint (30 min). Plasma corticosterone was measured using the ImmuChem Double Antibody Corticosterone 125I RIA kit (MP Biomedicals). Corticosterone concentration was calculated using AssayZap software (Biosoft).

**Patch-clamp recordings of IL-PFC neurons**

Male Sprague Dawley rats (postnatal days 25–32) were anesthetized deeply with sodium pentobarbital (30 mg/kg) and perfused with ice-cold dissection solution consisting of the following (in mM): 250 sucrose, 2.5 KCl, 25 NaHCO3, 1.0 NaH2PO4, 6 MgCl2, 0.5 CaCl2, and 25 glucose (bubbled with 95% O2/5% CO2). The brain was removed rapidly, blocked, and immersed in low-melting-point agarose (3% in above solution; Invitrogen). Transverse slices (300 µm) containing the IL-PFC (Paxinos, 2005) were cut using a Vibroslice tissue slicer (HA-752; Campden Instruments). The slices were placed in a chamber filled with oxygenated dissection solution for 30 min and then allowed to recover for >1 h at room temperature (RT) in an oxygenated aCSF solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.0 NaH2PO4, 1.0 MgCl2, 2.0 CaCl2, and 25 glucose. After recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments), mounted on the stage of an upright microscope (BX51WI; Olympus), and perfused with oxygenated aCSF at a rate of 3–6 ml/min at 31–33°C. Patch electrodes were constructed from thin-walled single-filamented borosilicate glass (1.5 mm outer diameter; World Precision Instruments) using a microelectrode puller (P-97; Sutter Instruments). Pipette resistances ranged from 3 to 5 MΩ, and seal resistances were >1 GΩ. Voltage-clamp recordings of EPSCs used an intracellular solution containing the following: 130 mM Cs-gluconate, 10 mM CsCl, 10 mM HEPES, 11 mM EGTA, 1.0 mM CaCl2, 2.0 mM MgATP, 1 mM QX-314, and 0.2% biocytin. IPSCs were recorded with an intracellular solution containing the following: 130 mM CsCl, 2 mM MgCl2, 10 mM HEPES, 2 mM Na2ATP, 0.4 mM Na3GTP, 1 mM QX-314, and 0.2% biocytin. Using infrared-differential interference contrast optics, layer V pyramidal neurons within the IL-PFC were located visually.
based on their large somata and the presence of a prominent apical dendrite extending toward the pial surface (Bacci et al., 2002). Whole-cell patch-clamp recordings were obtained from these neurons using a Multiclamp 700B amplifier (Molecular Devices) at a holding potential of -70 mV. Synaptic responses were evoked electrically (10–35 µA, 100 µs at 0.10 Hz) via a second patch electrode placed within layer II/III, which was connected to a constant-current stimulator (Master-8; A.M.P.I.). AMPAR-mediated EPSCs were isolated pharmacologically in the presence of 25µM AP-5, 10 µM gabazine, and 0.5 µM strychnine, whereas GABAAR-mediated IPSCs were recorded in the presence of 25 µM AP-5, 10 µM NBQX, and 0.5 µM strychnine. After 3 min of baseline recording, NPY (1µM) was bath applied for 5 min. Selection of 1 µM NPY concentration was based on a number of previous in vitro electrophysiological studies that used this concentration to examine potential effects on the excitability of cortical, hippocampal, and amygdalar neurons in brain slices (Bacci et al., 2002; Giesbrecht et al., 2010; Tu et al., 2005). Membrane voltages were adjusted for liquid junction potentials calculated using JPCalc software (P. Barry, University of New South Wales, Sydney, Australia; modified for Molecular Devices). Currents were filtered at 4 – 6 kHz through a -3 dB, four-pole low-pass Bessel filter, digitally sampled at 20 kHz, and stored on a personal computer (ICT) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.2 software; Molecular Devices).

Histology and immunohistochemistry

Brains were collected after perfusion with 4% paraformaldehyde for verification of cannula placement and immunohistochemistry.Brains were cut at 30 µm on a sliding microtome through forebrain prefrontal regions, and the resulting sections were stored in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at -20°C until processed for immunohistochemistry or histology. The following primary antibodies were used for double- immunofluorescence NPY (1:3000; Immunostar), NPY receptor subtype Y1 (1:50; Immunostar), glutamic acid decarboxylase 67 (GAD67; 1:1000; Millipore), and
calcium/calmodulin-dependent kinase II (CaMKII; 1:1000; Abcam). Sections were transferred from cryoprotectant to 50 m (KPBS; pH 7.2; 40 mM potassium phosphate dibasic, 10 mM potassium phosphate monobasic, and 0.9% sodium chloride) at RT. Cryoprotectant was rinsed (five times for 5 min) in KPBS, and the sections were transferred to KPBS plus 1.0% H2O2 and incubated for 10 min at RT. Sections were then washed (five times for 5 min) in KPBS at RT and placed in blocking solution [50 mM KPBS, 0.1% bovine serum albumin (BSA), and 0.2% Triton X-100] for 1 hr. at RT. Sections were incubated overnight (48 h for Y) at 4°C in primary antibody diluted in blocking solution. The following day, sections were rinsed in KPBS (five times for 5 min) and incubated in biotinylated anti-rabbit (or anti-mouse) secondary antibody (Vector Laboratories) diluted 1:500 in KPBS plus 0.1% BSA for 1 h at RT. Sections were rinsed in KPBS (five times for 5 min) and then treated with avidin–biotin complex (Vector Laboratories) at 1:1000 in KPBS plus 0.1% BSA for 1 h at RT. After this incubation, sections were rinsed again in KPBS (five times for 5 min) and subsequently incubated in biotin-labeled tyramide (PerkinElmer Life and Analytical Sciences) at 1:250 in KPBS with 0.3% H2O2 for 10 min at RT. Sections were rinsed in KPBS (five times for 5 min) and incubated in Cy3-conjugated streptavidin (Jackson ImmunoResearch) diluted 1:500 for 1 h at RT on a shaker in the dark. For double labeling, sections were rinsed (five times for 5 min) in KPBS and then incubated in the second primary antibody diluted as indicated in KPBS plus 0.1% BSA. After KPBS rinses (five times for 5 min), slices were then incubated in Cy5-labeled secondary antibody (Jackson ImmunoResearch) diluted 1:500 in KPBS plus 0.1% BSA at RT for 1 h covered. Sections were rinsed five times for 5 min in KPBS at RT after the final antibody incubation, mounted onto Superfrost Plus slides, and coverslipped with Gelvatol.

Sections were imaged using an AxioImager Z1 microscope (Zeiss) equipped with apotome (Z-stack) imaging capability (Axiocam MRm camera and AxioVision Release 4.6 software; Zeiss). All images were collected using a 20X air objective lens. Cy3 was excited using the 568 nm, whereas 647 nm was used to collect images of Cy5-labeled cells.
Colocalization was determined by overlapping signals. Images from the IL-PFC (3.2 to 2.20 mm from bregma) were acquired as Z-stacks. Flattened images were examined using NIH Image J software to quantify the total number of Y1-positive cells and the number colocalized with GAD67 or CaMKII using the cell counter tool. For immunohistochemistry data (Table 1), n=5 rats were used (three slices per animal). Brightness and contrast of the photomicrographs presented here were adjusted using Adobe Photoshop CS2 (Adobe Systems) to ensure the highest quality images for publication.

**Statistical analysis**

Histological assessment was performed on cresyl stained sections, and animals were included in statistical analysis if injector tracts were clearly visible within the stereotaxic coordinates defining the IL cortex (reported n for each group represents “hits”). Behavior scored manually by individuals blinded to treatment was used for analyses. Total seconds freezing during each tone presentation is expressed as a percentage of total tone presentation time. Data were analyzed using two-way repeated-measures ANOVAs (treatment X trial) or using one-way ANOVA or t-test (unpaired) when comparing mean percentage freezing for specific trial blocks between treatments (Prism 5; GraphPad Software). Repeated-measures ANOVA was used to analyze HPA response after restraint stress over blocks of time. Mean immobility counts in the FST test and error scores for the DWS test for aCSF and NPY cohorts were analyzed using unpaired t test. Post hoc comparisons were performed using the Holms–Sidak analysis.

Synaptic currents were analyzed using Clampfit (Molecular Devices) software, and mean current amplitudes were calculated using 1 min bins. Data are expressed as means ± SEMs. Normalized current amplitudes were compared between NPY and control (aCSF) conditions using two-way ANOVA. n refers to the number of neurons sampled in a given group unless specified otherwise. Prism software was used to analyze all data (GraphPad Software).
Results

IL NPY infusion compromises extinction consolidation leading to impaired retrieval of extinction memory

To evaluate the role of NPY on extinction acquisition and memory, we infused NPY into the IL subdivision of the mPFC 30 min before extinction training on Day 2 (Fig. 1C). This timing of intervention was chosen to evaluate NPY effects on fear expression, extinction learning, and memory. An NPY dose–response study showed no effects of NPY on Day 2 fear expression and extinction training as indicated by similar levels of freezing between NPY and aCSF groups (Fig. 1D). There was a significant effect of trial block (F(9,247) = 18.93; p < 0.05) but no effect of treatment (F(3,247) = 0.65; p > 0.05). However, a significant impairment of extinction memory retrieval was observed on Day 3 in rats infused with 10 and 30 pmol NPY but not at the 3 pmol dose (Fig. 1D, right). Repeated-measures ANOVA revealed a main effect of treatment (F(3,255) = 12.83; p < 0.05), trial (F(9,225) = 20.99; p < 0.05), and a significant trial X treatment interaction (F(27,255) = 1.833; p < 0.05). Post hoc comparisons confirmed that 10 and 30 pmol NPY-infused rats showed significantly higher levels of freezing during trial blocks 2–4 (p < 0.05) but no significant effects at 3 pmol NPY.

To confirm that NPY effects on retrieval were attributable to impaired consolidation of extinction and not a state-dependent phenomenon, we infused NPY (10 pmol) immediately after extinction training (Fig. 1E). Post-extinction NPY infusion also resulted in significantly higher freezing during extinction retrieval compared with aCSF-infused rats (Fig. 1F). Repeated-measures ANOVA revealed a significant effect of treatment (F(1,10) = 16.04; p < 0.05), trial block (F(9,80) = 13.37; p < 0.05), and a trend in trial X treatment interaction (F(9,80) = 1.917; p = 0.068), confirming that NPY compromises the consolidation of extinction.
NPY effects on extinction memory retrieval are mediated via the NPY Y1R

Selective Y1R antagonist BIBO was infused 10 min before NPY (10 pmol) administration on Day 2 (Fig. 2A). To investigate the role of tonic endogenous NPY on extinction memory, a separate cohort of rats was infused with BIBO alone without NPY. No significant differences in freezing were noted between groups during acquisition of extinction (Fig. 2B). Freezing was significantly attenuated during retrieval of extinction in NPY rats treated with BIBO (Fig. 2B). Two-way ANOVA revealed a significant main effect of treatment ($F(3,297) = 9.18; p < 0.05$) and trial ($F(9,297) = 26.72; p < 0.05$). BIBO-treated NPY rats showed equivalent levels of freezing as the control aCSF group ($F(1,198) = 0.81; p > 0.05$), suggesting that Y1R antagonism was sufficient to block the effects of NPY on extinction memory. Interestingly, animals treated with BIBO in the absence of NPY showed no significant difference in freezing compared with aCSF-treated animals ($F(1,164) = 0.51; p > 0.05$). As shown in Figure 2C, mean freezing in the NPY-treated group during initial trial blocks 1–4 was higher than control, BIBO alone, and BIBO plus NPY groups (one-way ANOVA; $F = 3.544; p < 0.05$). Post hoc analysis revealed significant differences between NPY and other treatment groups ($p < 0.05$).

NPY infusion into the IL cortex does not regulate neuroendocrine stress responses, depression-like behavior, or working memory performance

No significant differences were observed between NPY- and aCSF-infused animals in corticosterone response after restraint stress at any time interval (Fig. 3A). Two-way repeated-measures ANOVA revealed a significant effect of time ($F(3,54) = 85.82; p < 0.05$), with no effect of treatment ($F(1,54) = 0.09; p > 0.05$). There was no significant difference between immobility scores of aCSF- and NPY-treated animals in the FST (Fig. 3B; $t = 0.8663; p > 0.05$ by $t$ test). Additionally, NPY IL infusion did not elicit any significant effects on across-phase errors ($t =
0.8356; p > 0.05) or within-phase errors (t = 0.3254; p > 0.05) during the retrieval phase of DWS testing in the RAM (Fig. 3C, D).

**NPY enhances synaptic inhibition onto IL-PFC pyramidal neurons**

To examine the effects of NPY on inhibitory and excitatory synaptic signaling within the IL-PFC, GABAAR-mediated IPSCs (Fig. 4A) or AMPAR-mediated EPSCs (Fig. 4C) were evoked in layer V pyramidal neurons via electrical stimulation in layers II–III. NPY (1 µM) led to a significant potentiation of IPSCs (Fig. 4A) that outlasted the duration of the NPY exposure itself (n = 11; p < 0.0001 compared with aCSF only; two-way ANOVA; Fig. 4B). No such increase in IPSC amplitude was observed in layer V pyramidal neurons during continued perfusion with aCSF (n = 5; Fig. 4B). In contrast, the bath application of NPY (1 µM) evoked a modest, but statistically significant, decrease in EPSC amplitude (n = 12; p<0.05 compared with aCSF only; two-way ANOVA; Fig. 4D). Control experiments involving aCSF perfusion revealed no consistent time-dependent changes in EPSC amplitude during the 20 –25 min recording period (n = 5; Fig. 4D). Collectively, the results suggest that NPY significantly shifts the functional balance between synaptic excitation and inhibition onto layer V pyramidal neurons in a manner that would be predicted to reduce action potential discharge in the output neurons of the IL-PFC.

**Expression of NPY and NPY Y1R on IL neurons**

As shown in Figure 5, the Y1R was predominantly expressed on cell soma with negligible fiber staining. Y1R expression was evident in excitatory pyramidal cells positive for CaMKII, a marker for projection neurons (Rostkowski et al, 2009) (Fig. 5A–C). CaMKII colabeled cells accounted for 60.5% of Y1R-expressing cells (Table 1). Y1 expression was also observed in a few scattered cells positive for GAD67, a marker for GABAergic interneurons (Fig. 5D–F). GAD67 colabeled cells represented 12.7% of Y1R-expressing cells (Table 1). We also observed a few
NPY- immunopositive cell soma that were positive for GAD67 (Fig. 5G–I). No colocalization was observed between NPY- and CaMKII-positive cells (data not shown).

Discussion

Using behavioral, electrophysiological, and immunohistochemical approaches, we found the following: (1) pre-extinction and post- extinction training infusion of NPY in the IL cortex impaired retrieval of extinction memory, leaving the acquisition of extinction intact; (2) this effect was mediated via the Y1R, the predominant NPY receptor subtype in cortical areas; (3) NPY application increased inhibitory synaptic transmission onto IL projection neurons; (4) neuroendocrine stress response, depression-like behavior, and working memory performance were not affected by IL NPY; and (5) in the IL cortex, NPY and Y1 receptors are expressed in inhibitory GABAergic neurons and excitatory pyramidal projection neurons, placing them in an optimal position for regulating excitatory/inhibitory neurotransmission.

Infusion of NPY in the IL cortex before and immediately after extinction training elicited a robust impairment of extinction retrieval via inhibition of extinction consolidation, suggesting inactivation of this region by NPY. This is not surprising given that NPY is an inhibitory neuropeptide with potent anti- excitatory actions in other brain regions, such as the hippocampus, amygdala, and neocortex (Bacci et al., 2002; Colmers & Bleakman, 1994; Giesbrecht et al., 2010a; Molosh et al., 2013b). Effects of NPY on extinction memory were mediated by the NPY Y1R subtype, which is abundantly expressed on projection neurons of the IL cortex (Fig. 5) and modulates NPY regulation of fear extinction in the amygdala (Gutman et al., 2008). Retrieval of extinction is an IL-mediated outcome, because inactivation by IL-directed lesions or infusion of muscimol or TTX (Gregory J Quirk & Mueller, 2008; Sierra-Mercado et al., 2011) compromises retrieval. Neuronal activity in the IL cortex during extinction affects the consolidation of extinction learning (Burgos-Robles et al., 2007) and appears critical for the expression of extinction memory. Optogenetic silencing of IL neurons during extinction had no
effects on within-session extinction but impaired recall after 24h (Do-Monte et al., 2015a), suggesting a recruitment of the IL cortex during extinction training. Similarly, our data imply that NPY-evoked inhibition of IL activity during training and consolidation phases of extinction lead to impaired retrieval.

IL glutamatergic networks are critically involved in plasticity during extinction learning (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2004). AMPAR/NMDAR activation during extinction training and consolidation is required for successful extinction retrieval after 24h (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009). Additionally, enhancement of inhibitory GABAergic neurotransmission (e.g., by muscimol) infused before acquisition of extinction (but not before retrieval) impairs retrieval of extinction memory (Do-Monte et al., 2015; Sierra-Mercado et al., 2011).

Given the relevance of inhibitory and excitatory neurotransmission in extinction memory, we investigated whether NPY could modulate excitatory/inhibitory signaling in IL neurons. Our electrophysiological results suggest a potent inhibitory effect of NPY on the excitability of layer V pyramidal neurons, as evidenced by a persistent increase in GABAAR-mediated IPSCs and a modest decrease in evoked AMPAR-mediated EPSCs, which agrees with previous observations in neocortical, BLA, and hippocampal pyramidal neurons (Bacci et al., 2002; McQuiston & Colmers, 1996; Molosh et al., 2013b). NPY is expressed in GAD- positive GABA interneurons (Fig. 5) and can regulate GABA release (Sun et al., 2003). Although additional investigation is required to identify the underlying mechanisms, these NPY- evoked changes in synaptic efficacy are predicted to decrease excitability in cortical circuits, leading to dampened IL output and an impairment of extinction retrieval.

Output from the IL cortex suppresses the freezing response to tone via projections to the intercalated cells of the BLA, which, in turn, regulate central amygdala control of freezing (Quirk & Mueller, 2008). Because Y1R antagonism completely blocked effects of NPY on extinction retrieval, it is likely that activation of the Y1R by NPY reduces the activation of these neurons.
during extinction learning and consolidation, thereby affecting extinction memory. Our immunohistochemical studies revealed Y1R expression on a subset of CaMKII-positive pyramidal projection neurons and GAD67-positive interneurons in the IL cortex. We also found that NPY expression was localized to inhibitory GAD67-positive interneurons as reported previously (Alho et al., 1988; Eriksdotter-Nilsson et al., 1987). Collectively, observed antagonism of NPY effects on fear by Y1R antagonist, expression of Y1R and NPY on pyramidal projection neurons, and modulation of synaptic transmission onto pyramidal neurons by NPY suggest that NPY and the Y1R are optimally positioned for controlling excitatory output from prefrontal circuits and regulating retrieval of extinction.

In addition to fear memory modulation, the mPFC plays an important role in regulating neuroendocrine and behavioral responses to psychogenic stressors and in cognitive performance (Caetano et al., 2012; Radley et al., 2006; Spencer et al., 2005). Recent studies report modulation of forced swim behavior and HPA activity by IL-specific knockdown of glucocorticoid receptors (McKlveen et al., 2013). Specific lesions to the IL cortex result in inhibition of HPA response to acute restraint stress, suggesting that this region activates HPA output (Radley et al., 2006). No effects of NPY infusion on post-restraint HPA response/recovery, immobility in the FST, and working memory retrieval errors suggest that increased IL NPY does not have generalized effects on stress response, depression-like behaviors, or cognitive tasks under our test conditions.

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compromise top-down regulation of regions such as the amygdala and result in impaired processing of fear memories, leading to increased vulnerability to anxiety disorders such as PTSD.

Our data contrasts with previous studies reporting attenuation of conditioned fear and facilitated extinction by NPY (Gutman et al., 2008; Lach & de Lima, 2013; Verma et al., 2012). Global NPY and NPY receptor knockout mice exhibit enhanced fear acquisition and conditioned fear, as well as impaired extinction (Verma et al., 2012). Because infusion of the Y1 antagonist alone did not have any behavioral effects in our study, the pre-frontal NPY system may not be recruited under tonic conditions tested in these studies. However, increased cortical NPY occurring as a result of NPY genetic polymorphisms (Sommer et al., 2010) or after exposure to chronic stress (McGuire et al., 2011), may lead to increased synaptic concentrations of NPY. Intracerebroventricular infusion of NPY attenuated conditioned fear and improved extinction, an effect mediated by Y1Rs in the BLA (Fendt et al., 2009; Gutman et al., 2008). Retrieval of extinction memory, a phenomenon regulated by the IL cortex, has not been investigated previously. Our results in conjunction with previous work suggest that fear regulatory effects of NPY are complex and depend on the modulation of excitatory/inhibitory tone by NPY in distinct fear circuits. Thus, NPY-mediated inhibition of BLA projection neurons (Giesbrecht et al., 2010) would reduce fear and improve extinction (Gutman et al., 2008), whereas NPY-mediated inhibition of IL projection neurons would compromise IL-mediated excitation of intercalated (ITC) GABAergic neurons and enhance fear. Regional disparity between PFC and amygdala NPY expression is supported by previous observations. In a rodent model of chronic traumatization-evoked sensitization of fear, differential effects on NPY expression are observed: low in the amygdala and high in PFC (McGuire et al., 2011). In this regard, NPY gene polymorphism rs14167 is associated with differential NPY mRNA expression in the brain: high in the PFC (Sommer et al., 2010a) versus low in other brain regions (Zhou et al., 2008). Differences in posttranscriptional processing, synthesis, degradation, or transport may result in these effects.
Importantly, both decreased amygdala NPY and increased PFC NPY will lead to compromised processing of fear memories.

Previous work has proposed a role of NPY in PTSD, albeit as a stress resiliency factor (Sah & Geracioti, 2013; Wu et al., 2011). Reduced CSF concentration of NPY is observed in combat-related PTSD subjects (Sah et al., 2009; Sah et al., 2014). Currently, information on regional concentrations of NPY in postmortem brain samples from PTSD subjects is lacking. Thus, it is not evident whether lower CSF NPY reflects a global reduction. Dynamics of NPY diffusion into the CSF compartment is not known but may contribute to regional differences. Our data also have implications for NPY therapeutics, especially with regard to fear memory regulation. NPY supplementation via intranasal route or hippocampal infusion is beneficial for stress-, anxiety-, and startle-related outcomes in rodent models (Cohen et al., 2012; L. I. I. Serova et al., 2013). It would be important to investigate IL NPY and extinction memory outcomes in these models. Regional disparities in NPY expression/function should be considered as therapeutic options for NPY to become available in humans. Although the current study focused on male rats, it will be of interest to investigate sex differences given the vulnerability of females to fear processing disorders such as PTSD (Inslicht et al., 2013). In conclusion, we report modulation of IL neuronal excitation and extinction memory by NPY. In individuals expressing rs14167 gene polymorphism or with a history of chronic stress exposure, elevated prefrontal NPY may contribute to IL hypoactivity, resulting in impaired extinction memory and increased susceptibility to anxiety disorders such as PTSD.
Table 1
Distribution of Y1R immunoreactivity on CaMKII-positive glutamatergic and GAD67-positive GABAergic neurons in the rat IL cortex (see Fig 5)

<table>
<thead>
<tr>
<th>Population Y1R cells</th>
<th>Double-Labeled Population</th>
<th>% Double-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>403 ± 34</td>
<td>248 ± 28 (CaMKII positive)</td>
<td>60.5 (CaMKII)</td>
</tr>
<tr>
<td>523 ± 19</td>
<td>65 ± 6 (GAD67 positive)</td>
<td>12.7 (GAD67)</td>
</tr>
</tbody>
</table>

Data shown are mean ± SEM (n = 5; 3 slices per animal)
NPY infusion into the IL cortex impairs retrieval of extinction. A, Representative illustration showing location of cannula placements in the IL cortex based on histological assessment. Coronal sections represent coordinates +3.2 mm (top) and +2.7 mm (bottom) relative to bregma. B, Image showing the spread of fluorescent labeled NPY (FAM-NPY) within the IL subdivision of the mPFC. C, Timeline of the experiment showing infusion of NPY before extinction training on Day 2. D, Freezing response to tone for rats infused with aCSF (n = 12) or NPY (3, 10, and 30 pmol/0.2 μl; n = 6–8). Rats infused with 10 or 30 pmol NPY showed significantly higher freezing compared with aCSF rats during extinction retrieval on Day 3, as noted by higher levels of freezing (right). No significant difference in percentage freezing was noted during conditioning (left) or extinction training (middle). E, Schematic for post-extinction training infusion of NPY. F, Infusion of NPY (10 pmol) into the IL post-extinction acquisition significantly impaired retrieval of extinguished fear compared with the aCSF-infused group (n = 7 per group). Data shown are mean ± SEM; *p < 0.05 versus aCSF and 3 pmol NPY (Fig. 1D) or aCSF (Fig. 1F) group. For conditioning, data on post-shock freezing for shocks 1–6 are shown. For extinction and retrieval, data from tones 1–9 are shown. Magnitude of freezing plateaued at these time points, and no additional changes in freezing were observed in subsequent trials. PL, Prelimbic cortex; CC, corpus callosum. BL represents baseline freezing during 2 min before the delivery of conditioning, extinction, or retrieval trials.
Fig. 2

Fig. 2: NPY Y1R subtype mediates effects of NPY on extinction memory. A, Timeline of experiment. Y1R antagonist BIBO was infused 10 min before NPY infusion 30 min before extinction training on Day 2. B, Freezing response of rats infused with aCSF (n = 7), NPY (10 pmol; n = 9), BIBO (200 pmol; n = 8), and BIBO and NPY (BIBO-NPY; n = 10). NPY-evoked impairment of extinction retrieval (increased freezing; right) was attenuated significantly in rats infused with BIBO to levels observed in the aCSF treatment group. Rats infused with BIBO by itself did not elicit any significant differences in freezing during retrieval of extinction compared with aCSF cohort. No significant differences in conditioning (left) or conditioned fear expression and extinction learning (middle) were observed between groups. C, Mean percentage freezing during trials 1–4 showed significantly increased freezing in the NPY treatment group compared with aCSF, BIBO-NPY, and BIBO-alone groups. Data shown are mean ± SEM. * p < 0.05 versus other groups; #p < 0.05 versus the BIBO-NPY group. For conditioning, data on post-shock freezing for shocks 1–6 is shown. For extinction and retrieval, data from tones 1–9 is shown. Magnitude of freezing plateaued at these time points, and no additional changes in freezing were observed for subsequent trials. BL represents baseline freezing during 2 min before the delivery of conditioning, extinction, or retrieval trials.
Fig. 3: Infusion of NPY into the IL cortex does not affect post restraint neuroendocrine stress response, depression-like behavior in the FST, or working memory performance in the RAM. A, HPA response to restraint stress. Blood was collected from the tail vein at 15, 30, 60, and 120 min from the time the animals were exposed to the stressor (aCSF, n = 10; NPY, n = 9). B, FST behavior test. Immobility scores over 5 min for aCSF-infused (n = 12) and NPY-infused (n = 11) rats in the FST. Scores during the initial 5 min are reported because of high immobility in both groups after this period. C, D, RAM memory test. Mean across-phase (C) and within-phase (D) errors during retrieval testing are reported (n = 10 per group). No significant difference was noted between the NPY- and aCSF-infused animals in HPA response, immobility in the FST, or mean across-phase errors or within-phase errors in the RAM test compared with aCSF-infused rats. Data are mean ± SEM (p > 0.05).
Fig. 4: NPY potentiates GABAergic inhibition onto layer V pyramidal neurons in the IL-PFC. A, Examples of IPSCs in a layer V IL-PFC neuron after focal electrical stimulation in layers II–III, before (Baseline) and after the administration of NPY (1 μm). B, NPY significantly enhanced IPSC amplitude in these neurons (aCSF, n = 5; NPY, n = 11; p < 0.0001; two-way ANOVA). C, Representative examples of AMPAR-mediated EPSCs in a layer V neuron either before (Baseline) or after bath application of NPY (1 μm). D, Plot of normalized EPSC amplitude as a function of time demonstrating a slight but significant reduction in EPSC amplitudes after NPY exposure (n = 12; p = 0.028; two-way ANOVA) compared with control conditions (i.e., continued perfusion with aCSF; n = 5).
**Fig. 5**

Colocalization of Y1R (A–F) and NPY (G–I) with CaMKII-IR or GAD67-IR cells in the IL cortex. Y1R-immunopositive cells (A) showed colocalization with CaMKII-IR cells (B) as seen in merged image (C). A few Y1R-positive cells also colocalized with GAD67-IR cells (E) as seen in merged image (F). NPY-IR cells (G) showed colocalization with GAD67-IR cells (H; see I for merged image). Double-labeled cells (arrows) and single-labeled CaMKII or GAD67 cells (arrowheads) are visible. Scale bar, 20 μm.
CHAPTER 2

Study 2:

Neuropeptide Y projections to the Infalimbic Prefrontal Cortex
Abstract

Background: NPY, a peptide transmitter is highly expressed in forebrain areas regulating stress and emotional behaviors. As demonstrated in the preceding section of this chapter, NPY in the IL subdivision of the prefrontal cortex regulates the retrieval of extinguished fear. NPY and NPY receptor, Y1 exhibit high expression within the PFC. Notably, an abundance of NPY immunoreactive fibers is observed. Currently, the origin of NPY input into the IL PFC is not known. Studies undertaken in this section aimed to identify sources of NPY innervation of the PFC using fluorogold (FG) retrograde tracing with immunohistochemistry.

Methods: The retrograde tracer FG was injected via iontophoresis into the infralimbic (IL) division of the prefrontal cortex. Dual label immunohistochemistry was performed using antibodies selective to FG and NPY to identify brain areas with NPY projections to the IL.

Results: FG+/NPY+ cells were identified primarily within the dorsal raphe nucleus, particularly the caudal portion of the dorsal subnucleus (DRD). Other areas that contained sparse FG+/NPY+ co-labeled cells were the IL and PL PFC and the ventral hippocampal CA1. No NPY innervation from other PFC projecting, stress and fear regulatory areas enriched in NPY such as the hypothalamus or the locus coeruleus was observed.

Conclusions: Our studies identified regions associated with NPY circuits innervating the IL subdivision of the PFC. The dorsal raphe nucleus appears to be a major source of NPY innervation into the IL. Raphe-infralimbic NPY circuits may participate in the regulation of emotional behaviors.
Introduction

The prefrontal cortex (PFC) is a well-known regulator of many neurological functions including: executive function (Déziel et al., 2015), learning and memory (Peters et al., 2013), spatial orientation (Zhou et al., 2016), response inhibition (Moorman & Aston-Jones, 2015), social behavior (Bicks et al., 2015), stress and HPA axis function (McKlveen et al., 2013), as well as fear (Do-Monte et al., 2015; Vollmer et al., 2016). Impaired functioning of the PFC has been associated with several psychiatric disorders such as depression (Chang et al., 2014; Rive et al., 2013), schizophrenia (Sakurai et al., 2015) and PTSD (Arnsten et al., 2015). Several neuroimaging studies have reported altered activation within prefrontal subdivisions in patients with Posttraumatic Stress Disorder (PTSD) (Liberzon & Sripada, 2007). PFC hypoactivity has also been correlated with compromised extinction and retrieval (Milad et al., 2009). The exact mechanisms that contribute to altered PFC function is not evident however, a shift in excitatory/inhibitory tone is thought to play a role.

Neuropeptide Y (NPY) is a 36-amino acid peptide inhibitory transmitter that is highly expressed in the PFC. In situ hybridization as well as immunohistochemical studies have localized expression of NPY mRNA and protein in cortical areas, particularly in the superficial (I-II) and deeper (V-VI) layers in humans (Caberlotto et al., 2000) and similar areas in the rat brain (Morris, 1989). NPY has net inhibitory effects on neuronal signaling in the amygdala (Giesbrecht et al., 2010; Molosh et al., 2013), hippocampus (Haas et al., 1987) and the neocortex (Bacci et al., 2002). In recent studies, we reported enhanced inhibitory postsynaptic currents (IPSCs) in IL projection neurons (Vollmer et al., 2016); also see Fig. 4 in previous section of Ch. 2). We also reported significant impairment in the retrieval of extinguished fear in rat infused with NPY in the IL prior to extinction training (Vollmer et al., 2016; also see Fig. 1 in previous section of Ch. 2). PFC NPY connectivity with other areas has not been investigated. A majority of the NPY-immunopositive staining within the in the prefrontal cortex is observed within a dense network of
fibers, suggesting influx from other brain areas. Identification of these areas is relevant as they may regulate the release, activity and function of prefrontal NPY.

In order to identify neuroanatomical substrates contributing to pre-frontal NPY to initiate characterization of PFC NPY connectivity and circuitry, we conducted tract tracing studies using the retrograde tracer FluroGold (FG) injected in the IL subdivision of the PFC. The IL has been reported to display differential connectivity and heterogeneity in afferent projections from other PFC subdivisions (Hoover & Vertes, 2007). Detailed assessment of FG⁺-NPY⁺ co-labelled cell bodies was performed within forebrain and hindbrain areas that have been previously reported to provide efferent projections to the IL.

Methods

Animals

Male Sprague-Dawley rats from Harlan (Indianapolis, Indiana) weighing 225-250 g upon arrival were singly housed throughout the experiment in a temperature/humidity-controlled room on a 12-hour/12-hour light/dark cycle. Food (Teklad; Harlan) and water were available ad libitum. All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Retrograde tracing of NPY projections to the IL cortex

Iontophoretic administration of FG into the IL.

The retrograde tracer, FG was injected following previously published procedures (Myers et al., 2014). Animals (n=5) were anesthetized with an i.p. injection (1 ml/kg) of ketamine (87%)/xylazine (13%) and placed into a stereotaxic apparatus for iontophoretic delivery of FG (2% in sterile saline; Fluorochrome, Denver, CO), utilizing aseptic techniques. Using a glass micropipette with 15µm internal diameter and backfilled with FG, injections were targeted to the
infralimbic (IL) division of the PFC. Coordinates from bregma were AP +2.7, ML +0.6, DV -4.0 (Swanson Brain Atlas, 3rd edition). Alternating current was applied at 5µA for 7 seconds on and 7 seconds off for 21-35 sec, for a total of 2-3 current pulses. Finally, the micropipette was slowly removed, skull hole was sealed with Gelfoam®, and incision site closed with sterile surgical sutures. Animals were allowed to recover and tracer incubated in the animal for 13 days and sacrificed thereafter.

**Tissue Collection.** Animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% sodium phosphate-buffered paraformaldehyde. Brains were removed and post-fixed in 4% sodium phosphate-buffered paraformaldehyde for 24 hours, then stored in 30% sucrose at 4ºC. Brains were sectioned on a microtome in 30-µm coronal sections and stored in diethylpyrocarbonate (DEPC)-treated cryoprotectant (500 ml 0.1M phosphate buffer, pH 7.3, 10 gm polyvinyl-pyrrolidone, 300 gm sucrose, 300 ml ethylene glycol, per 1L of solution) until processed for immunohistochemistry.

**Immunohistochemistry**

Sections spanning the entire brain were processed for dual label immunohistochemical staining for FG+/NPY+ cells. An anti-NPY antibody, NPY02, directed against the mature form of the NPY peptide (generously provided by Dr. Eric Grouzmann, University Hospital of Lausanne, Lausanne, Switzerland) was used. This antibody has been previously characterized and reported to preferentially label NPY expressing cell bodies (Grouzmann et al., 1992; Leitermann et al., 2016). FG-positive soma were detected using an anti-FG antibody (generously provided by Dr. Stan Watson, University of Michigan, Ann Arbor, MI) which has been extensively characterized previously (Campeau and Watson, 2000).

Free-floating sections stored in DEPC-treated cryoprotectant solution following sectioning, were rinsed 5 times with 50 mM potassium phosphate buffered saline (KPBS)
solution. Following all incubations, sections were rinsed with 50 mM KPBS 5 times unless otherwise indicated. Slices were then incubated in blocking solution containing 50 mM KPBS with 10% normal donkey serum (NDS) and 0.25% TX-100 for 1 hr. at room temperature. After rinsing, slices were then incubated overnight in blocking solution containing rabbit 1° antibody against FG (1:500). On day 2, slices were rinsed and incubated with Cy3-conjugated donkey anti-rabbit 2° antibody (1:500; Jackson ImmunoResearch, West Grove, PA) in 1% NDS and 0.25% TX-100 for 1 hr. Following rinses, slices were incubated with a mouse 1° antibody against NPY (NPY02; 1:2500) in 3% NDS and 0.25% TX-100 overnight. On the morning of day 3, slices were rinsed then incubated with Alexa488-conjugated donkey anti-mouse 2° antibody (1:500; Jackson ImmunoResearch) in 3% NDS and 0.25% TX-100 for 1 hr. Sections were rinsed 4 times in 50 mM KPBS and mounted in 50 mM potassium phosphate-buffered solution and 1% gelatin onto ultrastick slides (Gold Seal) and coverslipped with polyvinyl alcohol anti-fading medium with DABCO (Sigma-Aldrich, St. Louis, MO).

For 3,3′-diaminobenzidine (DAB) staining, the same procedure was followed on day 1. On day 2, slices were rinsed, then incubated in biotinylated anti-rabbit secondary antibody (1:500, Vector Laboratories, Burlingame, CA) in 50mM KPBS + 1% NDS for 1 hr. at room temperature on a shaker. Following rinses, slices were incubated in ABC reagent (1ul A + 1ul B /1 ml in 50 mM KPBS + 1% NDS) for 1 hour on a shaker. Slices were rinsed then incubated in BT reagent (1:250, Perkin Elmer) for 10 min on a shaker. Slices were then rinsed and again incubated in ABC reagent for 1 hr. on shaker. Following rinses, slices were incubated in 0.05% DAB solution (Sigma, St. Louis, MO) with 0.015% H₂O₂ for approximately 5 min. To stop the DAB reaction slices were rinsed thoroughly in 50 mM KPBS. Slices were then rinsed 3 times for 5 min in KPB to remove salts. Slices were mounted in 50 mM potassium phosphate-buffered solution and 1% gelatin onto ultrastick slides (Gold Seal) and coverslipped using DPX mountant (Electron Microscopy Sciences, Hatfield, PA).
Detection of FG⁺/NPY⁺ cells

An initial assessment of IL hits-misses was performed and only those animals that had FG injection site localized exclusively within the IL (n=5) subdivision of the PFC were processed for further analysis (see Fig. 1 for representative images). Dual labeled FG and NPY (FG⁺/NPY⁺) immunopositive cells were localized rostro-caudally using fluorescence microscopy (Zeiss, Jena, Germany). The rat brain atlas (Paxinos, 2005) and previous comprehensive studies of projections to the IL and PL (Hoover & Vertes, 2007) were used as a guide. Images were acquired using the z-stack function (Axiovision, Zeiss). Images were cropped and brightness and contrast were adjusted using Photoshop (Adobe, San Jose, CA) and Image J (NIH, Bethesda, MD). Co-localization of FG and NPY was quantified in areas where FG⁺/NPY⁺ cells were visualized.

Results

Retrograde Tracer placement in the IL-PFC

Figure 1 shows a representative FG injection site in the IL. Only animals with injection sites that were majority located in the IL were included in further analysis.

NPY and FG antibody immunolabeling

Figure 2 illustrates staining patterns for the two antibodies we used in our studies: NPY02 (detects mature NPY) and the anti-FG antibody. Our labelling is consistent with previous studies performed with these antibodies (Grouzmann et al., 1992; Leitermann et al., 2016). NPY02 was used for detection of dual labeled FG⁺/NPY⁺ cells in this study due to its preferential labeling of NPY+ cell bodies.
FG immunostaining: immunofluorescence and DAB detection

Although immunofluorescence was employed for the detection of FG+/NPY+ dual-labelled cells, we also performed DAB immunohistochemistry, a more sensitive immunostain to verify the extent of FG tracing and whether the retrograde tracer reached hindbrain areas. We observed similar rostro-caudal FG staining pattern between immunofluorescence and DAB (Fig. 2). In 2 out of 5 rats (FG6 and FG10), we observed FG+ve cells in the locus coeruleus suggesting that in these animals the label successfully migrated to hindbrain areas.

NPY projections to the IL

In general, retrogradely labeled (FG-ir) cells were observed throughout the brain in patterns consistent with regions previously reported to project to the PFC (Hoover & Vertes, 2007). Similarly, NPY+ve cells and fibers were observed in several forebrain as well as hindbrain areas previously characterized by others (de Quidt & Emson, 1986) (Fig. 2). Dual stained FG- and NPY- positive cells were confined to selected areas of the brain as described below.

Dorsal Raphe Nucleus (DRN)

NPY immunoreactivity was seen throughout the rostral-caudal extent of the DRN, and a large portion of this immunoreactivity was in fibers (Fig. 3). FG+ve cells were also observed in the DR. Co-localization of FG+/NPY+ cells were observed in animals FG6 and FG10 that displayed highest levels of FG transport to the brainstem. Several FG+ve cells devoid of NPY immunoreactivity were observed and vice versa. In general, FG labeled cells were largely confined within the caudal portion of the DRN, and this is where co-labeling with NPY was observed, particularly in the dorsal subnucleus (DRD). The largest proportion (4/10, 40%) of NPY+/FG+ cells occurred in FG-6. Animal FG-10 also displayed NPY+/FG+ positive cells (3/8, 37.5%).
**Other brain regions**

FG+/NPY+ cells were also observed in other areas although there were less NPY+/FG+ cells present than in the dorsal raphe. These regions include the IL (FG6 and 10) and PL (FG6 and 8) PFC, as well as the ventral hippocampal CA1 (vCA1) subfield (FG6, 7, and 10) (Fig. 4). Several regions with known projections to the IL PFC that regulate stress, fear, and anxiety and are reported to have NPY expressing cells were examined. However, no FG+/NPY+ cells were observed in the basolateral amygdala (BLA), hypothalamic nuclei or locus coeruleus (LC). These areas contained numerous NPY immunoreactive cells as well as FG+ve cells, however no dual-labelled cells were observed.

**Discussion**

Despite the abundant expression of NPY and NPY receptors, their functional role in cortical areas has not been investigated. As described in the previous section of this chapter and (Vollmer et al., 2016), NPY and NPY Y1 receptors in the IL cortex regulate the retrieval of extinguished fear, an IL-mediated behavior of relevance to PTSD. However, very little is known about NPY circuits within, or projecting to, the PFC. This information is essential to understand mechanisms underlying NPY function in the PFC. The IL primarily shows a dense plexus of NPY fibers and comparatively low number of cell bodies, leading us to hypothesize that additional sources of NPY innervation to the IL may exist.

Using tract tracing, the current study shows the heterogeneity of NPY input to the IL PFC. NPY expressing cortical interneurons may represent one source of NPY in the IL. Previous studies have reported high expression of NPY mRNA within the cortex (de Quidt & Emson, 1986). Within the IL, NPY is expressed in a subpopulation of GAD67-positive interneurons (Vollmer et al., 2016). However, extensive characterization and phenotyping of
NPY expressing neurons in the IL has not been undertaken, and it is likely that more than one type of NPY expressing interneurons may exist in this area.

In the current study, we observed a small number of FG+/NPY+ cells within the IL and PL subdivisions, suggestive of local NPY innervation within cortical circuits. As our study demonstrated, other sites also send NPY projections to the infralimbic cortex. The predominant, novel NPY projection was identified from the dorsal raphe to the IL.

**NPY projections from the DRN to the IL PFC**

We observed FG filled cells with NPY immunoreactivity in 2 of the 5 animals (FG6 and 10) that received FG injections in the IL. These animals possibly had the best uptake of the tracer to brainstem areas and were the only two animals to show FG filled cells in brainstem areas, including the locus coeruleus (LC). It is also possible that injection sites differed in their location in different layers of the IL that may have led to observed variability in transport destinations.

Studies using Phaseolus vulgaris leucoagglutinin (PHA-L), an anterograde tracer, injected into the DR have shown moderately dense projections from both the rostral and caudal DRN specifically to the IL, and these projections were concentrated mostly in layers I/II and V/VI of the IL (Vertes, 1991). Functional connectivity between medial PFC and DR has been shown previously. High frequency stimulation (HFS) of the IL decreases the cellular firing of DR neurons that may regulate transmitter release in projection fields (Srejic et al., 2015). We have observed NPY+ fibers throughout all layers of the IL PFC, but have not identified these as coming from either local interneurons or outside brain areas. We were not able to identify layer specificity of the DR NPY projection due to the widespread nature of our FG injections in the IL, which hit, at least in part, all layers of the IL.
Due to the low number of animals (2 out of 5) that contained double labeled cells in the DR these conclusions should be viewed as preliminary. A higher “n” is need to further validate these findings.

**NPY projections to the IL PFC from the ventral hippocampal CA1**

In addition to the DR, double-labeled cells were observed in the vCA1 subfield. Though there were fewer NPY/FG double labeled cells than the DRN, there were more single-labeled NPY+ve and FG+ve cell bodies within the vCA1. Double labeled cells were observed in this area in 3 out of the 5 animals (FG6, 7, and 10), but only a small number (1-2) were observed per animal.

Previous studies examining areas that project to the IL have shown a dense projection from the vCA1 to the IL (Hoover & Vertes, 2007). Functional connectivity between these two areas has been displayed to be important in multiple studies. Lesions of one side of the PFC along with the ventral hippocampus in the opposite hemisphere (essentially disconnecting these two areas from each other) leads to both impulsive and compulsive behavior in rats (Chudasama et al., 2012). Optogenetic Inhibition of ventral hippocampal input to the mPFC has been shown to significantly increase open arm time in the EPM and center time in open field, as well disrupt phase locking of the firing of mPFC neurons with ventral hippocampal theta oscillations, which is representative of aversion (Padilla-Coreano et al., 2016). Though this circuit is well known to regulate aversion, impulse control, and anxiety, it is unlikely that the small number of NPY+ve cells that project from the vCA1 to the IL-PFC have a very significant contribution to this circuit. As with the DRN, we were not able to identify layer specificity of the vCA1 to IL NPY projection due to the widespread nature of our FG injections in the IL.

**Lack of NPY input to the IL PFC from the basolateral amygdala (BLA) and locus coeruleus (LC)**
The BLA and LC are areas well known to regulate fear and stress responses (Perusini et al., 2016; Reyes et al., 2015; Sabban et al., 2015; Schmeltzer et al., 2015) and are known to project to both the IL and PL PFC (Hoover & Vertes, 2007). For this reason, we hypothesized these two areas as potential sites for NPY projections to the PFC. However, though FG filled cells were observed in the BLA (particularly the basal region and the adjacent dorsal endopiriform nucleus (DEn) and amygdolostriatal transition areas (ASTr) and the LC in most animals, no co-localization with NPY was observed. The AStr was of particular interest due to its recent discovery as a primary area of NPY projection to the BLA. In all IL injected animals both FG and NPY immunoreactivity was observed in the AStr, however no co-localization was observed. The connection between the BLA and PFC has been demonstrated to be very important for the extinction of fear (Tovote et al., 2015) and reciprocal connections between these two areas have been observed by previous tracing studies (Hoover & Vertes, 2007; Vertes, 2004).

Similarly, high NPY immunoreactivity was observed in the LC, and although FG positive cells were observed in the LC and adjacent sites, no co-localization was noted in any of the 5 animals. Thus, NPY innervation to the PFC likely stems from medial brain stem sites such as the raphe and to a lesser extent from the ventral hippocampal formation.

**Functional implications**

Both the PFC (McKlveen et al., 2013; Rive et al., 2013) and the DRN (Heller, 2016; Michelsen et al., 2007) have been implicated in the regulation of stress, depression and anxiety. Specifically, glutamatergic projections from the PFC target the DR, and blocking the NMDA receptors in the DR has been shown to attenuate learned helplessness behavior in rats in the forced swim test (Grahn et al., 2000). It is possible that NPY projections into the PFC from the DR will represent a negative feedback mechanism to reduce glutamatergic stimulation of the DR by the PFC during response to an uncontrollable stressor.
NPY+/FG+ cells were observed only in the caudal DR in this study. The caudal subregion of the DRN is important in translating stress into emotional behaviors (McDevitt et al., 2011). Compared with other DRN subregions, the caudal DRN is particularly sensitive to uncontrollable stress (Grahn et al., 1999), and serotoninergic mechanisms within this region are known to regulate the expression of fear-related behavior (McDevitt et al., 2011). Evidence implicates PFC-DRN interactions in regulating the behavioral control of stress (Amat et al., 2005). Thus, activation of raphe neurons was dependent on the PFC under controllable stress conditions impacting behavioral choices and outcomes. The DRN sends reciprocal serotonergic projections to the PFC that are activated following aversive exposures such as inescapable shock (Bland et al., 2003). Given our observations of NPY in DRN-PFC circuits, it would be important to investigate the association of NPY and serotonin within the raphe and whether stress exposure regulates the DRN mediated release of NPY in the PFC.

Studies on DRN NPY have been highly limited. NPY is present in moderately high concentrations in DR (O’Donohue et al., 1985), and NPY binding sites are also evident in this region (Martel et al., 1990). Previous electrophysiological studies have shown inhibition of 5-HT mediated responses in the DR by NPY (Kombian & Colmers, 1992). In addition to 5-HT, neuropeptides such as CRH in the DR is implicated in mediating the behavioral consequences of uncontrollable stress (Hammack et al., 2003). NPY-CRH interactions within the raphe may also play an important role in stress-evoked behavior. Collectively, our data indicates a novel NPY projection pathway from the DRN to the IL that may be relevant in the behavioral outcomes of stress. More in depth studies are needed to identify the cellular targets and functional role of this DRN-IL NPY projection.
**Figure 1:** FluoroGold injection sites in the IL-PFC. (A) Representative injection site in the IL-PFC (anti-FG DAB staining). (B) Spread of FG injections into the IL-PFC for animals used for tract tracing studies, largest (yellow) and smallest (blue). FG, FluorGold.
Figure 2: Representative staining patterns for anti-FG and NPY02 antibodies throughout the brain of animal FG6. Green dots, FG; red dots, NPY02; orange dots, co-labeled (FG+/NPY02+). Each dot represents 1 immunopositive cell body.
Figure 3: NPY projection from the caudal DRD to the IL-PFC. (A) Colocalization of NPY02 and FG in the caudal DRD. (B) DAB staining for FG confirming the caudal DRD to IL-PFC projection. (C) Atlas section showing the area where FG+/NPY+ cells were found. Blue box indicates area shown in pictures. DRD, dorsal raphe nucleus dorsal subregion; IL-PFC, infralimbic prefrontal cortex; FG, FluorGold.
Figure 4: Other areas with NPY+ cells projecting to the IL-PFC. (A) IL-PFC (B) PL-PFC (C) ventral hippocampal CA1. Red-NPY, Green-FG. IL-PFC, infralimbic prefrontal cortex; PL-PFC, prelimbic prefrontal cortex; FG, FluorGold.
Chapter 3
Neuropeptide Y in the amygdala: regulation of PTSD relevant behaviors
Abstract

Background: PTSD is a disorder that involves dysregulated trauma-associated emotional memories. The neurobiology of PTSD has therefore focused on brain regions regulating fear and anxiety, primarily the amygdala, a key site for acquisition and storage of fear memory. Neuroimaging studies have shown increased activation of the amygdala and its correlation with compromised extinction of fear in PTSD subjects. However, biological factors promoting amygdala hyperactivity and their association with fear regulation is not known. Recent studies report a primary role of inhibitory mechanisms within the amygdala in regulating the extinction of fear. NPY, an inhibitory peptide transmitter is reduced in PTSD patients. NPY is highly expressed in the amygdala and regulates excitation within this region. Studies in this chapter investigate the effects of NPY modulation in the amygdala on PTSD-relevant behaviors, primarily the extinction of conditioned fear.

Methods: Pharmacological (study 1) and lentiviral (study 2) techniques were used to manipulate NPY in the amygdala. We focused on the basolateral nucleus (BLA), a sub region that is known to regulate fear and anxiety. Under study 1, bilateral cannulas were targeted to the BLA in adult rats. NPY or artificial cerebrospinal fluid (aCSF) was infused 30 min. prior to extinction training in a cued-fear conditioning paradigm. Under study 2, a lentiviral construct (CMV-NPY) was characterized for overexpression of NPY in the amygdala. Adult male rats were administered CMV-NPY or reverse oriented control (CMV-rNPY). Effects on PTSD-relevant behaviors: anxiety, acoustic startle, and fear conditioning were assessed under baseline conditions and following chronic variable stress (CVS).

Results: Study 1: NPY infusion in the BLA prior to extinction training significantly decreased conditioned freezing to the tone, as well as facilitated extinction. Study 2: NPY overexpression in the BLA had no significant effect on anxiety-like behavior and acoustic startle response.
Unexpectedly, delivery of NPY lentivirus and its expression in capsular areas surrounding the BLA enhanced chronic stress evoked increase in conditioned fear and compromised extinction.

**Conclusions:** NPY within the amygdala has a site-specific effect on fear related behavior. Increased NPY into the basolateral division of the amygdala has fear-reducing effects and facilitates extinction. However, increased expression within the external capsular area enriched in intercalated cells may enhance fear and compromise extinction. Given previous evidence of NPY in multiple sites within the amygdala, the peptide may exert fear-enhancing or reducing effects dependent on localization within fear circuits.
Introduction

The amygdala is a key brain structure regulating fear acquisition and storage, supported consistently by a large body of evidence using various experimental paradigms (Ehrlich et al., 2009; Fanselow & Poulos, 2005; LeDoux, 2000; Maren, 2001). Dysregulated processing of trauma associated memories is seen in individuals inflicted with post-traumatic stress disorder (PTSD) where persistently elevated fear responses to stimuli associated with the traumatic event are observed as a result of compromised extinction and recall of extinction (Milad et al., 2009). Several neuroimaging studies report a significant correlation of extinction deficits with hyperactivity of the amygdala (Schaefer et al., 2002). Interestingly, damage to the amygdala was reported to be effective in protecting from PTSD (Koenigs et al., 2008). Recently, a primary role of inhibitory mechanisms within the amygdala in the regulation of fear memory have been demonstrated (Aseed et al, 2015; Ehrlich et al., 2009 and references therein). Compromised balance of excitation/inhibition within the amygdala may result in improper processing of fear memories. The basolateral subdivision of the amygdala (BLA) has been shown to be particularly important for regulation of fear memories (Helmstetter & Bellgowan, 1994; LeDoux et al., 1990; Wilensky et al., 1999).

In recent studies we reported reduced concentration of inhibitory peptide transmitter, neuropeptide Y (NPY) in cerebrospinal fluid (CS)F collected from combat veterans with PTSD, as well as, an inverse correlation of CSF NPY with intrusive symptoms associated with trauma associated memories (Sah et al., 2014). NPY is expressed within the amygdala in various sub-nuclei such as the BLA, CeA and within intercalated cell masses (ITCs) surrounding the BLA (Wood et al., 2015). Previous studies have reported inhibition of fear-associated startle (Gutman et al., 2008), decreased conditioned fear and facilitated extinction by NPY administered in the amygdala (Fendt et al., 2009; reviewed in Tasan et al., 2016). Previously we reported a
significant reduction in amygdalar NPY concentration in rats during recovery from chronic variable stress that was associated with exaggerated fear reinstatement and fearful arousal (McGuire et al., 2010, McGuire et al., 2011). Collectively, these evidences support a potential contribution of NPY in the regulation of fear memory. Given its role in increasing synaptic inhibition within the amygdala, we hypothesized reduced conditioned fear and facilitated extinction in NPY supplemented animals. Our primary target for NPY intervention was the basolateral nucleus given its central role in the regulation of fear memories (Helmstetter & Bellgowan, 1994; LeDoux et al., 1990; Wilensky et al., 1999).

Two approaches were employed for NPY manipulation: pharmacological delivery of NPY (study 1) and lentivirus-mediated delivery of NPY (study 2). In the first study, we implanted bilateral cannulas targeting the BLA to deliver NPY in rats tested in a cued fear conditioning paradigm. NPY effects on conditioned fear and extinction were assessed. Study 2 utilized a lentivirus for modulation of NPY expression within the BLA. Effects on anxiety-like behavior, acoustic startle responses and conditioned fear expression and extinction were investigated. Effects of NPY overexpression were also assessed in animals exposed to chronic variable stress (CVS). Collectively, our data showed significant fear modulatory effects of NPY that appear to be site-specific. While administration in the BLA resulted in attenuated conditioned freezing and facilitated extinction, enhanced NPY expression in capsular areas surrounding the BLA significantly enhanced conditioned freezing in stressed rats. Thus, fear-modulatory effects of NPY in the amygdala are complex. Given the pivotal role of the amygdala in PTSD pathophysiology further studies using cell- and circuit-specific approaches are warranted to investigate contributions of NPY in this region.

Methods

Subjects. Male Long Evans rats (Harlan, Indianapolis, Indiana) weighing 225-250 g upon arrival were singly housed throughout the experiment in a temperature/humidity-controlled room on a
12-hour/12-hour light/dark cycle. Food (Teklad; Harlan) and water were available ad libitum. All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Study 1 Procedures**

**Surgery**

Rats were habituated to the vivarium for 1 week after arrival. For cannula surgery, rats were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg) and surgically implanted with bilateral stainless steel cannulae (Plastics One) aimed at the BLA using the following coordinates from bregma (anterior-posterior: −2.6 mm; mediolateral: ±5.0 mm; dorsoventral: −8.4 mm from skull). Protection and patency of the cannulae were maintained by inserting obturators between microinjections. Rats were allowed to recover for 2 weeks and handled daily during the second week, before any behavioral manipulations were performed.

**Intracranial NPY administration**

The timing of NPY administration on Day 2 was prior to the measurement of conditioned freezing and extinction training given a primary role of the BLA in the regulation of both conditioned fear and extinction. NPY (10 pmol; Bachem, Bupendorf, Switzerland) or artificial CSF (aCSF) was administered 30 min before placement of the animal in the test chamber. The 10 pmol NPY dose was selected as it has been used previously for NPY effects on fear potentiated startle extinction and stress via the BLA (Gutman et al., 2008; Sajdyk et al., 2008).

**Cued Fear Conditioning**

A 3-day fear conditioning paradigm consisting of training (D1), conditioned fear expression and extinction (D2) and extinction retrieval (D3) was used as described previously (Quirk et al., 2000) with minor modifications (see Fig. 1 for schematic, same paradigm was used in all fear conditioning studies). On D1, rats were placed in the chamber (Clever Sys, Reston, VA) and after 2 minutes received 5 habituation tone trials (80 dB, 30 sec) with an inter-trial interval of 2
min. Following habituation, animals received 7 tones that co-terminated with a footshock (0.6 mA, 0.5 sec.). On D2, animals were placed in the training context and exposed to 15 tone-only trials. On D3, animals were placed in the same context and exposed to 10 tone-only trials. As reported previously by us and others (Quirk et al., 2000; Vollmer et al., 2016) this paradigm produces conditioned freezing responses to tone on D2 (and no freezing to context prior to exposure to tone). Freezing, defined as an absence of movement except that needed for breathing was scored using Freezescan software (Clever Sys Inc.) and confirmed by hand scored videos by an observer blinded to experimental groups. Statistical analysis was performed using Freezescan data. The chambers and enclosures were cleaned between rats with 10% ethanol.

**Study 2 Procedures**

**Lentiviral constructs**

The viral vectors used for this study were purchased from the University of South Carolina Viral Vector Core (constructs manufactured by Dr. Boris Kantor). The NPY expression vector, pLenti-NPY-IRES-GFP (CMV-NPY) and a reverse oriented control virus pLenti-rNPY-reverse orientation-IRES-GFP (CMV-rNPY) were characterized (see Fig. 2 for vector maps). These constructs utilize the cytomegalovirus (CMV) promoter and contain a green fluorescent protein (GFP) tag for detection of virus delivery into cells. Based on recommendations from Dr. Kantor, the following titers were used for in vitro and in vivo studies (1.7 x 10^9 iu/mL for CMV-NPY and 1.1 x 10^9 iu/mL for CMV-rNPY). Prior to in vivo administration viruses were tested *in vitro* using the 4B immortalized hypothalamic cell line that have been reported to express NPY in previous studies (Nedungadi et al., 2013).
In Vitro

Cell Culture and Transfection

4B cells (Dr. Toni Pak, Loyola University, Chicago, Illinois) were seeded in HyClone Dulbecco’s modified Eagle’s medium (DMEM)/high glucose media (with L-glutamine and L-glucose; Thermo Scientific, Waltham, Massachusetts) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia). Cells were grown in large culture flasks, treated with trypsin (Invitrogen, Carlsbad, California) and were seeded in 6-well plates at a density of 0.5 x 10^6 cells/ml in 2ml media. Following an overnight incubation cells reached a confluenc of ~80%, and were processed for transfection. Media was removed and replaced with 2.5μL PBS containing CMV-NPY vector (1.7x10^9 iu/mL), control CMV-rNPY vector (1.1x10^9 iu/mL), or PBS, diluted in 2 mL of growth media. After 24 hours, the contents of the wells were aspirated and media was replaced (2 mL). Cells were harvested 3 days later for quantification of NPY and GFP mRNA using quantitative polymerase chain reaction (qPCR). Alternatively, cells used for immunohistochemistry were grown on coverslips in 12 well plates. Cell density and virus titer was reduced and adjusted for surface area (half the amount was used for 12 well plates). After 3 days, media was removed and cells were fixed on the coverslips using 4% paraformaldehyde for 20 min and processed for immunocytochemistry.

Real Time Quantitative Polymerase Chain Reaction (RT qPCR)

RNA from cells was isolated using an RNeasy kit, according to manufacturer protocol (Qiagen, Valencia, California). The RNA quantity and quality were determined with a NanoVue Plus spectrophotometer (General Electric Healthcare, Piscataway, New Jersey). The collected RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove genomic DNA and reverse transcribed with a SuperScript III complementary DNA synthesis kit according to manufacturer protocol (Invitrogen, Carlsbad, CA). RT qPCR analysis was performed in an iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad). Primers for NPY mRNA (10 μmol/L)
(forward: 5'-TACTCCGCTCTGCAGACACTACATC-3'; and reverse: 5'-CACATGGAAGGTCTTCAAGCC-3'), GFP (10 μmol/L) (forward: 5'-AGCAAAAGACCCCAACGAGAA-3'; and reverse: 5'-GGCGGCGGTCACGAA-3'), and the housekeeping gene L-32 (forward: 5'-CATCGTAGAAAGAGCAGCAG-3'; and reverse: 5'-GCACACAAGCCATCTTATTCCAT-3') were used (Integrated DNA Technologies, Coralville, Iowa). Quantification of complementary DNA was determined with iQ SYBR Green Supermix (Bio-Rad). Values were calculated with L-32 as an internal standard. NPY mRNA expression is presented as a percentage of expression in control PBS exposed cells. Negative RT samples were included to rule out genomic DNA contamination.

**Immunocytochemistry**

Following removal of media and incubation with 4% paraformaldehyde for 20 min. coverslips were rinsed with 50 mM KPBS 3 times. Coverslips were then incubated in 500µL blocking solution (1.0% bovine serum albumin (BSA), 0.3% Triton X100 in 50 mM KPBS) for 45 min. Block was removed and coverslips were then incubated in primary antibody against GFP (1:1000, Life Technologies, Carlsbad, CA) or NPY (1:3000, Immunostar, Hudson, WI) for 1.5-2 hours. Following primary antibody incubation coverslips were rinsed 3 times with 50 mM KPBS. Cover slips were then incubated in secondary antibody Alexa488 (GFP) or Cy3 (NPY) donkey anti-rabbit (1:500, Jackson ImmunoResearch, West Grove, PA) for 45 min. Coverslips were rinsed 3 times in 50 mM potassium phosphate-buffered solution and allowed to dry in the dark. Once dry, one drop of Vectatsheild mounting media with Dapi (fluorescent stain that binds strongly to A-T rich regions in DNA) (Vector Laboratories, Burlingame, CA) was applied to each coverslip and they were inverted onto ultrastick slides (Gold Seal, Portsmouth, NH) and stored in a cold room until pictures were taken using an inverted fluorescent microscope.

**In vivo**

**Subjects**
Male Long Evans rats (Harlan, Indianapolis, Indiana) weighing 225-250 g upon arrival were singly housed throughout the experiment in a temperature/humidity-controlled room on a 12-hour/12-hour light/dark cycle. Food (Teklad; Harlan) and water were available ad libitum. All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Stereotaxic Surgery.** Following one week of habituation, animals were anesthetized (87 mg/kg ketamine, 13 mg/kg xylazine), and preemptive analgesia (butorphanol) and antibiotic (gentamicin) were administered. Animals received 0.5 μL bilateral microinjections into the BLA (anterior-posterior [AP] = -2.6, medial-lateral [ML] ± 5.0, and dorsal-ventral [DV] = -8.4, (Paxinos, 2005)) of CMV-NPY vector (n=5,10) or CMV-rNPY control vector (n=5,9) in PBS with a beveled, 25-gauge, 2-μL Hamilton syringe (Reno, Nevada). To reduce tissue damage, each injection took place over 5 min. After the needle was lowered to the coordinates, it remained in place for 5 min. The virus was infused over 5 min with a micro driver (Model 5001; Kopf, Tujunga, California) and remained in place for 5 min to allow for complete diffusion. Animals recovered for 72 hours in BSL2 housing and were then moved to their normal housing room for the rest of the recovery time (at least 2-3 weeks) before any experiments.

**Chronic Variable Stress (CVS)**

CVS was comprised of twice daily (AM and PM) repeated and unpredictable stressors, including cold swims (10 min, 16–18ºC), warm swims (20 min, 30–32ºC), cold room exposure (1 hour, 4ºC), shaker stress (1 hour, 100 rpm), open field + novel object exposure (novel object was a red circular weight, 5 min), hypoxia (30 min, 8% oxygen), elevated zero maze (EZM, 5 min.), and overnight housing in a mouse cage (overnight crowding). The first exposure to cold swim (morning of D2) and open field + novel object (morning of D5) were videotaped and scored for anxiety and depression-like behaviors during chronic stress.
Open Field

Animals were placed in a 72 x 72 cm field made of white plastic with 36 cm walls surrounding each side for 5 min. A video recording of the behavior of the animal was scored and analyzed with Clever TopScan Software (CleverSys, Reston, Virginia). Time spent in the center versus the periphery of the open field was used as a measure of anxiety-like behavior (Belzung & Griebel, 2001). Each animal was placed in the same place, facing the center of the open field. The open field was cleaned with 10% EtOH between each animal. Behavior was recorded and scored by an observer blinded to the experimental condition. The same procedures were used during the open field stressor during CVS.

Acoustic Startle Response (ASR)

Startle response to an unexpected acoustic stimulus was measured using the SR-LAB startle response system (San Diego Instruments, San Diego, CA). The apparatus included ventilated, soundproof chambers measuring approximately 52 cm x 52 cm x 76 cm and contained an enclosure of approximately 12.5 cm diameter to keep the rat over the sensor. The enclosure was of sufficient size to restrict but not restrain the rat and allowed it to turn around. The chambers were calibrated using the SR-LAB standardization unit (San Diego Instruments, San Diego, CA), which transmits a precise series of pulses to the sensor located on each enclosure, allowing each chamber to be adjusted to the same read-out value for an identical stimulus prior to testing. To further minimize any effect of measurement differences between the chambers, an equal number of animals from each experimental group were tested in each chamber. The chambers and enclosures were cleaned between rats with 10% ethanol. The test consisted of 30 trials. Background noise in the chamber was maintained at 68 dB. The acoustic stimulus was a 40 ms, 108 dB burst of white noise emitted at intervals determined semi-randomly by computer. Inter-stimulus intervals were between 3 and 30 s with a minimum step between interval lengths of 3 s. Movement inside the tube was detected by a piezoelectric accelerometer below the frame. For each trial, measurements were taken at 1 ms intervals for a response
window of 150 ms following the startle stimulus using National Instruments Data Acquisition Software (San Diego Instruments, San Diego, CA). The maximum voltage change (Vmax) within the recording window over the averaged baseline (5 ms) just prior to stimulus was used for data.

**Fear Conditioning**

All trials had a 2 min. inter-trial interval unless otherwise noted. Freezing was defined as an absence of movement except what is needed for breathing. Freezing was scored using the Clever Sys software and confirmed by a blind observer that visually hand scored the videos. The chambers and enclosures were cleaned between rats with 10% ethanol. For cannulation studies, a 3-day fear conditioning paradigm was used. On D1, rats were placed in the chamber (Clever Sys, Reston, VA) and after 2 min. received 5 habituation tone trials (80 dB, 30 sec.). Following habituation, animals received 7 tones that co-terminated with a footshock (0.6 mA, 0.5 sec.). On D2, animals were placed in the chamber, and exposed to 15 tone-only trials. On D3, animals were again placed in the chamber and exposed to 10 tone-only trials.

For overexpression studies, a 4-day paradigm was used. D1-3 were the same as the cannulation study fear conditioning paradigm. On D4, animals were again placed in the chamber. Animals received 3 tone-only trials, 2 min. following the last tone-only trail, animals received a tone trial that co-terminated with a reminder shock (0.6 mA, 0.5 sec.). 2 min following the reminder shock, animals receive another 3 tone-only trials in order to investigate reinstatement of fear. Trials to extinction were calculated by finding at which trial animals froze below 10% and maintained that level of freezing for at least 3 more trials.

**Tissue Collection.** Animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% sodium phosphate-buffered paraformaldehyde. Brains were postfixed in 4% sodium phosphate-buffered paraformaldehyde for 24 hours, then stored in 30% sucrose in water at 4°C. Brains were sectioned on a microtome in 30-μm coronal sections.

**Immunohistochemistry**
Tissue sections were immunolabeled with primary antibodies against green fluorescent protein (GFP) (1:1500, Life Technologies) and NPY (1:3000; Immunostar), with standard immunohistochemical procedures. All fluorescent secondary antibodies were from Jackson Immuno Research (West Grove, PA). Free-floating sections stored in DEPC-treated cryoprotectant solution following sectioning, were rinsed 5 times with 50 mM potassium phosphate buffered saline (KPBS) solution. Following all incubations, sections were rinsed with 50 mM KPBS 5 times unless otherwise indicated.

For NPY-GFP co-labeling, slices were incubated in 0.3% hydrogen peroxide in 50 mM KPBS for 10 min. on a shaker at room temperature. Following rinses until no bubbles were remaining, slices were incubated in blocking solution (0.5% BSA, 0.2% TX-100 in 50 mM KPBS) for 1 hr. Slices were incubated overnight in anti-NPY primary antibody at 1:3000 dilution in blocking solution. The following morning, slices were rinsed and incubated in secondary antibody (Cy3 donkey anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, PA), for 1 h at room temperature. Slices were rinsed and incubated in blocking solution for 1 hr. Slices were then incubated overnight in rabbit anti-GFP antibody at 1:1500 dilution in blocking solution. The following day, slices were rinsed and incubated in secondary antibody Alexa488 donkey anti-rabbit (1:500; Jackson ImmunoResearch).

To mount sections on slides, they were rinsed 4 times in 50 mM KPBS and mounted in 50 mM potassium phosphate-buffered solution and 1% gelatin onto ultrastick slides (Gold Seal) and coverslipped with polyvinyl alcohol anti-fading medium with DABCO (Sigma-Aldrich, St. Louis, MO).

Results

Study 1

BLA NPY reduces conditioned fear and enhances extinction
To evaluate the role of NPY on conditioned fear, extinction acquisition and memory, we infused NPY into the BLA subdivision of the mPFC 30 min before extinction training on Day 2 (Fig. 1A schematic). To assess whether all animals trained similarly in the conditioning paradigm freezing was measured on D1. As shown in Fig. 1B, there were no significant difference in freezing between NPY and aCSF cohorts across trials. A two way repeated measures ANOVA revealed a significant effect of trail block (F(11,143)=56.36 p<0.05) but no treatment (F(1,13)=0.18, p>0.05) or treatment x trial interaction (F(11,143)=0.48, p>0.05). Conditioned freezing (trial 1-2) as well as freezing during extinction trials on day 2 revealed reduced freezing in NPY treated rats in comparison with aCSF treated animals (Fig 1C). Repeated measures ANOVA revealed a main effect of treatment (F(1,13)=16.42, p<0.05), trial block (F(11,143)=5.108, p<0.05) and a trial x treatment interaction (F(11,143)=2.33, p<0.05, Fig. 2B). NPY treated rats showed significantly reduced conditioned freezing, as well as significantly decreased freezing during extinction trials (Fig. 1D). Post hoc Tukey’s test confirmed that rats infused with NPY showed significantly lower freezing on trials 1-4 (p<0.05).

Study 2 Experiment 1

Functional Characterization of NPY Lentivirus in vitro

In order to ensure the efficacy of our NPY overexpression virus, we first performed in vitro experiments to investigate the effects of viral transfection on NPY mRNA and protein expression. We transfected immortalized, hypothalamic 4B cells with either CMV-NPY, CMV-rNPY, or PBS treatment as a control. This cell line was selected given previous evidence from our lab (K. Eaton, unpublished observations) of NPY expression, supporting the existence of NPY transcriptional and translational mechanisms in these cells. Measurement of NPY mRNA by qPCR revealed significantly increased expression of the NPY transcript in CMV-NPY transfected cells compared with control CMV-rNPY vector and PBS treated cells (Fig. 3A). Immunocytochemical staining was performed to confirm NPY protein expression in transfected
cells. As shown in Fig. 3C, CMV-NPY infected cells had higher NPY immunoreactivity than CMV-rNPY treated cells. Similar GFP and DAPI immunostaining was observed between NPY and rNPY viral vector transfected cells, suggesting that transfection efficiency and cell viability may be comparable between the two (Fig. 3B). However, this conclusion is qualitative and based on a visual assessment.

Next, we validated the ability of CMV-NPY to increase NPY expression in vivo. Viral constructs were targeted to the BLA. As shown in Fig. 4B, ANOVA revealed higher NPY immunoreactivity was observed in CMV-NPY transfected animals (F(2,13)=11.16, p<0.05). There was also increased co-labeling of NPY in GFP positive cells in CMV-NPY injected animals compared to CMV-rNPY animals (see representative pics in Fig. 4C).

**NPY overexpression in the BLA has no significant effects on body weight and food intake**

NPY is an established modulator of food intake and weight gain via the hypothalamus (reviewed in Loh et al, 2015). Although NPY overexpression in amygdalar nuclei is not expected to modulate these outcomes, we verified the effects of BLA NPY overexpression on weight gain or food intake. We began measurement after 7 days of recovery from stereotaxic surgery. As shown in Fig. 5, no effect of virus treatment was observed on weight gain (F(1,22)=0.06, p>0.05) or food intake (F(1,22)=0.28, p>0.05).

The BLA is an important site for the regulation of anxiety-like behavior. Modulation of anxiety-like behavior was tested in the open field test in animals treated with NPY overexpression virus, CMV-NPY, control CMV-rNPY vector or PBS. As shown in Fig. 6B, CMV-NPY injected rats showed a significant increase in center field entries (1 way ANOVA, F(2,44)=3.772, p<0.05, Fig. 6A), however, no significant differences were seen between groups
for center time (1 way ANOVA, F(2,44)=0.74, p>0.05, Fig. 4C) and latency to center (1 way ANOVA, F(2,44)=1.14, p>0.05, Fig. 4D). There was no significant difference in distance travelled (1 way ANOVA, F(2,44)=2.94, p>0.05, Fig. 4E) between groups, indicating there was no effect of NPY overexpression in the BLA on locomotion in the open field. Collectively, our data revealed attenuation in anxiety-like behavior represented by increased frequency of center field visits, however, this anxiolytic effect may be considered mild in the absence of significant effects on center preference or time.

Given the relevance of the amygdala as a site for sensorimotor gating, we measured acoustic startle behavior. Repeated measures ANOVA showed an effect of trial block for both Vmax (F(5,110)= 3.90, p<0.05) and average (F(5,110)=3.34, p<0.05) startle, but no effect of virus treatment on Vmax (F(2,44)= 2.48, p>0.05) or average (F(2,44)=1.59, p>0.05) or a trial x treatment interaction for Vmax (F(10,220)=1.61, p>0.05) or average (F(10,220)=1.50, p>0.05) startle, indicating no effect of NPY overexpression within the BLA on startle reactivity (Fig. 7).

**Study 2 Experiment 2**

**Validity of CMV-NPY administration and effects on NPY expression**

Using GFP immunolabeling, we assessed the location of lentivirus transfection sites within the amygdala (Fig. 8). As evident, a majority of injections were delivered in the external capsular area of the basolateral amygdala, contrary to our expectation. We also assessed NPY expressing cells in this area between CMV-NPY and CMV-rNPY treated animals (Fig. 8C). As shown, significantly higher numbers of NPY+ cells were observed in the CMV-NPY treated animals compared with the CMV-rNPY (Students t test, t(18)=3.99, p<0.05). CMV-rNPY control groups include all hits within amygdala nuclei, as there were no significant differences observed in any measured behavior between control virus hits in different subnuclei of the amygdala.
Effects of NPY overexpression in the lateral ITC area on chronic stress-evoked behavior (anxiety-like, startle and depression-like behaviors)

Rats with CMV-NPY or CMV-rNPY transfection were exposed to a 1 week (7 day) chronic variable stress paradigm. Effects on anxiety-like, depression-like behaviors, acoustic startle response, fear conditioning and extinction were assessed. Exposure to CVS produced a significant decrease in body weight in both CMV-NPY and CMV-rNPY treated groups as compared with control handled rats. Repeated measures 3-way ANOVA using treatment x stress x time as variables produced a significant effect of stress \((F(1,22)=10.53, p<0.05)\) and time \((F(3,66)=745.89,p<0.05)\), but no effect of treatment \((F(1,22)=0.20, p>0.05)\) (Fig. 9B).

Exposure to the forced swim test (FST) revealed no significant difference in immobility between ITC-NPY and CMV-rNPY treated cohorts (Students t-test, \(t(10)=p>0.05\), Fig. 9C). Exposure to the open field also revealed no differences in behavioral response between ITC-NPY and CMV-rNPY treated groups. Unpaired t test revealed no significant difference in center time (Student’s t-test, \(t(10)=00.14, p>0.05\)), Latency to center (Student’s t-test, \(t(8)=1.16, p>0.05\)) and center entries (Student’s t-test, \(t(10)=0.72, p>0.05\)). Motor activity during the open field exposure also showed no significant difference between groups (Fig. 10B-E).

Following a 7-day recovery CVS exposed and control groups were tested for acoustic startle response. Repeated measures ANOVA for showed significant effects of trial block \((F(5,110)=2.34, p<0.05)\), but no effect of stress \((F(1,22)=1.32, p>0.05)\) or virus treatment \((F(1,22)=1.61, p>0.05)\) for Vmax startle (Fig. 11B). In addition, there was a significant effect of
Lentiviral NPY overexpression in the area of the capsular intercalated cells: effects on conditioned fear and extinction

Acquisition comprising of training to tone-shock exposures revealed no significant differences between groups (Fig. 12B). Three-way repeated measures ANOVA using stress x treatment x trial as variables showed a significant effect of trial (F (11,242)=67.59, p<0.05), but no effect of stress (F(1, 22)=2.30, p>0.05) or virus treatment (F(1, 22)=0.68, p>0.05) was observed indicating that all groups had comparable freezing to the tone-shock pairing suggestive of consistent learning among groups. On D2, exposure to 15 tones in the absence of shock revealed interesting differences in conditioned freezing and extinction between groups. Animals treated with CMV-NPY lentivirus in the lateral capsular area elicited higher levels of freezing as compared with the control CMV-rNPY treated groups (Fig. 12C). CMV-NPY animals exposed to CVS had highest freezing that was significantly different from CMV-rNPY treated control and CVS treated groups. Repeated measures ANOVA revealed a significant effects of treatment (F(1,22)=16.51, p<0.05), time (F(14,308)=24.70, p<0.05) and treatment X time interaction (F(1,308)=4.38, p<0.05). Post hoc analysis showed that freezing during trial 1 representing conditioned fear was significantly higher (p<0.05) in both NPY lentivirus treated CVS and no stress control cohort as compared with no NPY control and CVS groups. Subsequent trials also revealed significant differences indicative of effects on extinction learning. The CVS exposed, CMV-rNPY (control vector) transfected rats showed higher freezing compared to the CMV-rNPY no stress group for trials 4 and 5 (p<0.05) reflective of deficits in extinction learning in CVS exposed animals irrespective of NPY treatment. In the CMV-NPY treated animals that were CVS exposed, this deficit was more robust (p<0.05 for trials 1-6 compared to no stress
controls). On D3, retrieval of extinction was tested between groups. As shown in Fig. 12D, CMV-NPY injected animals, especially those exposed to CVS, showed higher freezing to tone as compared with other groups. There was a significant effect of trial \( (F(9, 81)=9.94, p<0.05) \) as measured by 3 way repeated measures ANOVA. However, there was no significant effects of stress \( (F(1,22)=1.02, p>0.05) \) or treatment \( (F(1,22)=1.80, p>0.05) \).

**Discussion**

The current study investigated the role of NPY in subdivisions of the amygdala on behaviors relevant to PTSD, specifically fear, anxiety and startle. Given the relevance of emotional dysregulation and persistent trauma memories in PTSD subject our primary focus was on the regulation of fear related behavior. Using pharmacological and lentiviral techniques we manipulated NPY in the amygdala. While the basolateral nucleus was our target for NPY intervention, we also accidently targeted the lateral paracapsular area of the BLA containing intercalated cells that yielded interesting data. Collectively, our findings reveal complex, site-specific effects of NPY on conditioned fear, extinction learning and retrieval.

Administration of NPY peptide directly into the BLA prior to extinction training significantly reduced conditioned freezing and facilitated extinction for multiple reasons. Several studies support an important role of BLA activation in fear, with increased activation correlating with increased fear learning (Hoffman et al., 2014; Izumi et al., 2011; Sengupta et al., 2016). Pharmacological inhibition of the BLA with muscimol results in significantly attenuated conditioned freezing and facilitated extinction and extinction retrieval. Fear regulatory effects of NPY have been demonstrated in previous studies using genetic models and global amygdala infusions (Fendt et al., 2009; A Thorsell et al., 2000; Verma et al., 2015). Most studies have reported a significant reduction in fear (freezing) to an auditory (but not contextual) conditioned fear stimulus as well as an increased rate of fear extinction to auditory stimuli. However, there is little information on sub-nuclei specific NPY modulation within the amygdala. Recent studies
have indicated a complex network of inhibitory-excitatory circuits involving activation/disinhibition of output neurons that participate in distinct aspects of fear expression and memory (Ehrlich et al., 2009; Paré et al., 2004). Most studies on NPY and NPY receptor expression and functional responses using slice electrophysiology have focused on the BLA. NPY shows high expression in the BLA along with the Y1 and Y2 receptor subtypes (Tasan et al., 2015). NPY receptors are expressed throughout the BLA, however the most highly expressed receptor subtype is Y1, which has a high degree (99.9%) of co-localization with CaMKII, a maker for projection neurons (Rostkowski et al., 2009). Studies of NPY effects in the BLA have shown it to have a net inhibitory effect on glutamatergic projection neurons. This occurs through reduction of NMDA-mediated excitatory post synaptic currents (EPSCs) and an increase in GABAA-mediated inhibitory post synaptic currents (IPSCs), which are mediated by protein kinase A (PKA) and exchange protein activated by cAMP (ePAC), respectively (Molosh et al., 2013a). BLA pyramidal neurons project to the central nucleus-medial division (CeM) output neurons that project to areas such as the hypothalamus and brain stem for behavioral and autonomic expression of fear (reviewed in Pape & Pare, 2010). Consistent with this, we observed a significant reduction of conditioned freezing as well as facilitated extinction suggesting that NPY may inhibit BLA activity leading to attenuated freezing (fear) behavior. These effects are possibly not state dependent, as animals treated with NPY still show attenuated freezing to the tone 24 hr. later despite not receiving an NPY injection before testing. Predator exposure has been shown to activate NPY-positive cells in the BLA; it remains to be explored whether this effect is long lasting as our animals were only tested at 30 min. and 24 hrs. following NPY injection into the BLA.

We also characterized a novel NPY-overexpression vector, CMV-NPY that successfully increased the expression of NPY transcript and protein in vitro and in vivo. Compared with other viruses tested by us, SPWG-NPY that uses the phosphoglycerate kinase-1 (PKG) promoter and leads to low GFP expression (data not shown), transfection with CMV-NPY yielded detectable
expression of GFP and NPY within the same cell that was relevant for our *in vivo* studies. It should be noted that the CMV promoter is present in most neurons, meaning our virus targeted both NPY expressing and non-NPY expressing neurons. Our lentivirus experiment findings were more complicated and dependent on the site of virus delivery and its potential impact on areas in the vicinity of the BLA. In the initial *in vivo* experiment, NPY overexpression was successfully targeted to the BLA, and produced very modest effects on anxiety (increased # of entries to the center square of the open field) as well as no significant effect on acoustic startle response. One study using recombinant adeno-associated viral (rAAV) vector to overexpress NPY in the whole amygdala found anxiolytic effects in the open field, elevated plus maze (EPM) and light-dark transition tests (Christiansen et al., 2014). However, it is hard to compare these effects to ours due to the widespread nature of the injection and the fact that the animals were food restricted in order to be weight matched with the control group, which may have been a stressor. Another study using a herpes-simplex type-1 (HSV) mediated vector to overexpress NPY in the whole amygdala found an increase in percent time spent in the open arms of the EPM, but no difference in open arm entries, making the data hard to interpret (Primeaux et al., 2005). Previous experiments on direct NPY injection into the BLA produced significant attenuation of anxiety-like behavior in social interaction test (Sajdyk et al., 2008). Pharmacological NPY intervention would be expected to activate NPY Y1 receptors producing more specific effects in comparison to chronic overexpression of NPY in cell types other than Y receptor expressing cells; a caveat in nonselective lentivirus transfection paradigms. The BLA has been identified as a site for sensorimotor gating and startle responses (Rajbhandari et al., 2015); via a BLA-nucleus accumbens-ventral palladium-pedunculopontine tegmental nucleus (PPTg)-pontine reticular formation (PnC) pathway that regulates startle and prepulse inhibition (Leumann et al., 2001). Recent studies have reported stress-evoked increase in corticotrophin releasing hormone (CRH) and noradrenergic tone within projection neurons in the BLA in the enhancement of startle response (Rajbhandari et al., 2015). Our lentivirus experiments indicate
that NPY may not be recruited in these effects; however, this result should be confirmed with direct pharmacological NPY intervention. In a study that examined NPY effects on startle, BLA NPY infusion was found to be effective at reducing fear-potentiated startle but not baseline startle responses, indicating no effect of BLA NPY on baseline startle responses (Gutman et al., 2008), consistent with our data.

In our chronic stress experiments, we observed interesting, although unanticipated, modulation of fear memory related behaviors by NPY overexpression in paracapsular cells surrounding the BLA. Historically, pyramidal projection cells within the BLA and CeA have been the focus in fear-regulation (Johansen et al., 2010). More recent work from quite a few labs have shown the relevance of inhibitory circuits in providing feed forward inhibition gating response output from BLA and CeA projection neurons (Ciocchi et al., 2010; Ehrlich et al., 2009; Haubensak et al., 2010; Paré et al., 2004). Thus, compromised inhibition within these circuits would enhance activation within projection neurons leading to enhanced fear expression. An important group of inhibitory “gate-keeper” cells are housed within paracapsular regions of the BLA, referred to as the ITCs. The two primary clusters are the medial ITCs (mITCs) between the BLA and CeA that gate input to the CeA; and the lateral ITCs (lITCs) situated within the external capsule that gate cortical input to the BLA from cortical afferents and project to BLA pyramidal neurons (Marowsky et al., 2005). This area primarily houses GABAergic interneurons constituting the main cell type (Duvarci & Pare, 2014; Nitecka & Ben-Ari, 1987; Paré & Smith, 1993) and has been reported to express NPY (Wood et al., 2015). Most of our animals received virus injections into the lITCs capsular division leading to increased NPY within this inhibitory locus. Interestingly, this intervention led to significant enhancement of conditioned fear, compromised extinction and extinction retrieval. Exposure to CVS in non-NPY manipulated rats led to deficits in extinction learning as reported earlier for chronic stress exposures (Miracle et al., 2006). However, CVS exposed rats with NPY overexpression within the lITCs elicited further increase in freezing behavior during conditioned fear expression as well as extinction.
Interestingly, NPY-overexpression in IITC without CVS exposure also elicited increased freezing suggesting that increased IITC NPY by itself can modulate conditioned fear and extinction. Previous studies have reported that modulation of excitation/inhibition of ITC cells can modulate conditioned fear and extinction. Pharmacological stimulation of beta 3 adrenoceptors selectively enhances the inhibitory projection from the external capsule ITC cells to BLA projection cells and attenuates both fear and extinction learning, showing a role for these cells in fear and extinction learning (Skelly et al., 2016). In contrast, dopamine, a transmitter mediating stress-evoked enhancement of fear inhibits cells within the lateral ITCs leading to disinhibition of BLA projection neurons (Marowsky et al., 2005).

Increasing the inhibitory tone of this ITC population by NPY may then lead to aberrant BLA function due to the ability of these cells to generate IPSCs in BLA projections cells (Marowsky et al., 2005). As inactivation studies have shown, these principal BLA projection neurons are essential for fear memory formation (Muller et al., 1997; Wilensky et al., 1999). Biophysical modeling shows that BLA principal projection neurons with higher intrinsic excitability are more likely to be incorporated into the fear memory trace (Kim et al., 2013). It can therefore be speculated that interfering with lateral ITC inhibition of BLA projection neurons may result in overall higher intrinsic excitability of these neurons and a stronger overall fear memory trace in the BLA, resulting in higher levels of fear behavior. Our observed behavior is consistent with these speculations, however, further studies on direct administration into the IITC area as well as NPY effects on IPSCs generated by these cells are required to confirm our behavioral results. It should again be noted that our virus targeted both NPY expressing and non-NPY expressing neurons. To more accurately gauge what impact NPY in the IITC area has on behavior, a more targeted viral approach should be used. Our data is particularly relevant to PTSD pathophysiology where impaired cortical control of the amygdala and hyperactivity of the amygdala have been implicated.
Collectively, results from this section suggest fear modulatory effects of NPY within the amygdala that appear to be site-dependent; while inhibition of projection neurons within the BLA can attenuate fear, inhibition of feed forward inhibitory circuits, and can lead to enhanced fear memory related behaviors. Thus, more circuit- and site- specific approaches are necessary to fully understand behavioral contributions of forebrain NPY.
Fig. 1: NPY infusion in BLA reduces conditioned freezing to a tone. (A) Study 1 timeline: Rats were trained in an auditory cued fear conditioning paradigm (see methods for details). 24 hr. post training animals were infused with NPY (10pmol/side) or aCSF 30 min prior to exposure to conditioning tones for extinction training. After 24hr rats were exposed to tones to measure retrieval of extinction. Freezing behavior was measured for training, extinction and retrieval phases. (B) Rats within the aCSF and NPY groups show no significant differences in freezing behavior during training to a cued conditioning paradigm involving exposure to tone-shock pairings. (C) Infusion of NPY into the BLA 30 min. before exposure to conditioning tones results in a significant reduction in conditioned freezing compared to the aCSF infused group. (C) No significant differences in freezing between aCSF and NPY treated groups were observed 24 hr. later during the extinction retrieval test. Data shown are mean +/- sem, *p<0.05 versus aCSF, n=7-8/group.
Fig. 2: Vector maps of NPY overexpression vector, CMV-NPY and the control reverse oriented vector, CMV-rNPY. The NPY cassette was inserted downstream of the Cytomegalovirus (CMV) promoter, followed by an internal ribosome entry site (IRES) that allows for translation initiation in an end-independent manner. Both vectors also contain an enhanced green fluorescent protein (eGFP) cassette. In addition, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was added to enhance expression of the transgenes. LTR, long terminal repeat.
Fig. 3

A. NPY expression \textit{in vitro}

![Graph showing NPY expression levels for CMV-NPY, CMV-rNPY, and PBS treatments.]

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Fig. 3: In vitro characterization of NPY-overexpression lentiviral construct in hypothalamic 4B cells. (A) Cells were transfected with CMV-NPY, CMV-rNPY or PBS. RNA was collected 72 hr. post transfection for quantitative PCR. A significant increase in NPY expression was observed in CMV-NPY transfected cells versus CMV-rNPY and PBS transfected cells. No statistically significant difference was observed between CMV-rNPY and PBS treated cells. Immunocytochemical detection of GFP (B) and NPY (C) immunoreactivity in CMV-NPY and CMV-rNPY transfected 4B hypothalamic cells and co-localization with nuclear cell stain, DAPI. Qualitative observation shows increased NPY immunopositive cells as compared with control vector transfected cells. DAPI, 4',6-diamidino-2-phenylindole, GFP, green fluorescent protein. *p<0.05 vs. CMV-rNPY and PBS), n=3 wells/treatment, run in triplicate.
Fig. 4: CMV-NPY lentivirus increases NPY expression in vivo. (A) Representative picture of virus localization within in BLA (B) Quantification of NPY immunoreactivity in the BLA of animals treated with CMV-NPY, CMV-rNPY, and PBS control. CMV-NPY produced an increase in percent area stained for NPY immunoreactivity. No significant difference was observed between CMV-rNPY and PBS groups. (C) Representative pictures of NPY and GFP immunostaining within the BLA of CMV-NPY and control CMV-rNPY virus treated animals, white arrows indicate double-labeled cells. *p<0.05 vs. CMV-rNPY and PBS. NPY, Neuropeptide Y, GFP, green fluorescent protein, PBS, phosphate buffered saline. Images were acquired at 10X magnification (A) or 20x magnification (C). n=6/group
Fig. 5: CMV-NPY or CMV-rNPY transfection into the BLA does not affect weight gain or food intake in rats. (A) Weight gain in virus and phosphate buffered saline (PBS) treated animals following 1-week recovery from lentivirus infusion surgery. (B) Daily food (standard rat chow) intake for virus and PBS treated animals following 1-week recovery from lentivirus infusion surgery. n=6-12/group
Fig. 6: Effects of CMV-NPY, CMV-rNPY, or PBS administration on anxiety-like behavior in the open field test (OF). (A) Study 2, Experiment 1 timeline: Lentivirus CMV-NPY or control CMV-rNPY virus was injected. Following 2 weeks of recovery animals were tested in the open field (OF) or acoustic startle response (ASR). Animals were sacrificed and perfused for stereotaxic assessment of hits and misses and NPY expression. (B) CMV-NPY significantly increased entries into the center area of the open field arena. There were no significant difference between groups in center time (C), latency to center (D) and motor activity (E). *p<0.05 vs. CMV-NPY and PBS, n=6-12/group.
Fig. 7

A. Lentivirus Surgery

(CMV-NPY, CMV-rNPY, or PBS)

Recovery | OF | ASR | Sac | 2d | 60-90 min

B. CMV-NPY | CMV-rNPY | PBS

Vmax Startle (mV)

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C. Average startle (mV)

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Fig. 7: CMV-NPY administration into the BLA does not regulate acoustic startle response. (A) Study 2, Experiment 1 timeline. (B) Vmax (maximum millivolt (mV) output generated from the piezoelectric sensor sealed to the underside of the enclosure) following startle stimulus. (C) Average (mV responses averaged over each trial block) Stimulus = 108 dB, 30 trials (each trial block represents 5 trials), ITI randomized (5-30 sec), n=6-12/group. OF, open field, ASR, acoustic startle response.
Fig. 8: Administration of CMV-NPY and control CMV-rNPY constructs into the external paracapsular area of the basolateral amygdala. (A) Representative picture showing GFP+ve cells within the lateral capsule area housing intercalated cell clusters, lateral ITCs. Right hand illustration shows the location of ITC cell clusters in paracapsular areas, inhibitory interneurons within these areas and their connections to other neurons within the fear circuit (from Ehlrich et al, 2009). (B) Representative images showing co-localization of NPY and GFP immunoreactivity in the lateral ITC cluster. Co-localized NPY and GFP immunoreactive cells were evident in animals treated with CMV-NPY (top three panels). (C) CMV-NPY injected animals show significantly increased number of NPY immunopositive cells within the lateral ITC area (*p<0.05 versus CMV-rNPY group; n=6 animals/group, 3 tissues sections per animal (bilateral quantification). NPY, Neuropeptide Y, GFP, green fluorescent protein. ITC, intercalated cells, mITC, medial ITCs, lITC, lateral ITCs, LA, lateral amygdala, BA, basal amygdala, CEm, medial central amygdala, CEI, lateral central amygdala.)
Fig. 9: Effects of lentiviral CMV-NPY and control vector CMV-rNPY administration in the lateral ITC area on body weight and immobility in the forced swim test. (A) Study 2, Experiment 1 timeline: Following recovery from injection of CMV-NPY or CMV-rNPY rats were either exposed to 7 days of chronic variable stress (CVS) or handled as controls. Anxiety-like (open field test, OF) and depression-like (forced swim test; FST) behaviors were assessed during CVS between stress groups. Following a one-week recovery all animals were exposed to acoustic startle response (ASR) and cued fear conditioning paradigm to measure conditioned fear, extinction and extinction retrieval. (B) Exposure to CVS lead to a significant loss in body weight as compared with no stress control groups. No significant differences in body weight were observed between CMV-NPY (NPY) and CMV-rNPY (Control) treatments. (B) CMV-NPY did not have a significant effect on immobility in the forced swim test. n=6-9/group.
Fig. 10: Effects of lentiviral CMV-NPY and control vector CMV-rNPY administration in the lateral ITC area on anxiety-like behavior in the open field. (A) Study 2, Experiment 1 timeline. No significant differences were observed in open field behaviors such as center entries (C), center time (D), latency to center (E), and motor activity (F). n=6-9/group. FST, forced swim test, OF, open field, ASR, acoustic startle response.
Fig. 11: CMV-NPY administration into the lateral ITC does not affect acoustic startle responses in CVS or control handled animals. (A) Study 2, Experiment 1 timeline. (B) Vmax (maximum millivolt (mV) output generated from the piezoelectric sensor sealed to the underside of the enclosure) following startle stimulus. (C) Average (mV responses averaged over each trial block) Stimulus = 108 dB, 30 trials (each trial block represents 5 trials), ITI randomized (5-30 sec), n=6-9/group. FST, forced swim test, OF, open field, ASR, acoustic startle response.
Fig. 12: CMV-NPY evoked NPY overexpression in the lateral ITC potentiates conditioned fear and impairs acquisition of extinction. CMV-NPY overexpressing animals are noted as NPY and CMV-rNPY animals are noted as Con for simplicity. (A) Study 2, Experiment 1 timeline. (B) Percent freezing during fear conditioning training phase did not reveal any significant effect of virus treatment or stress exposure. (C) Percent freezing was significantly higher in NPY virus groups (NPY-Con and NPY-CVS; shown as triangles) as compared with control virus treated groups (Con-Con and Con-CVS, shown as squares). Significantly higher freezing was noted during trials 1-2 representing conditioned fear expression. NPY virus treated animals also showed significantly higher freezing during extinction acquisition trials as compared with control and CVS exposed groups. Of note, control virus treated animals exposed to CVS (Con-CVS) also showed significantly higher freezing during extinction suggesting impaired acquisition of extinction. (D) Percent freezing during extinction retrieval session 24h later showed no significant differences between groups. *p<0.05 vs. Con-Con, #p<0.05 vs. Con-CVS; n=6-9/group. FST, forced swim test, OF, open field, ASR, acoustic startle response.
Chapter 4

Effects of chronic versus acute stress on fear memory and startle: regulation of forebrain NPY

Abstract

Background: The chronicity of trauma exposure plays an important role in the pathophysiology of posttraumatic stress disorder (PTSD). Thus, exposure to multiple traumas on a chronic scale leads to worse outcomes than acute events. The rationale for the current study was to investigate the effects of a single adverse event versus the same event on a background of chronic stress. We hypothesized that a history of chronic stress would lead to worse behavioral outcomes than a single event alone.

Methods: Rats were exposed to either a single traumatic event in the form of foot shocks (acute shock, AS), or to footshocks on a background of chronic stress (chronic variable stress-shock, CVS-S). PTSD-relevant behaviors: fear memory and acoustic startle responses were measured following 7d recovery.

Results: In line with our hypothesis, CVS-S elicited significant increase in fear acquisition and conditioning versus the AS group. Surprisingly, CVS-S elicited reduced startle reactivity to an acoustic stimulus in comparison with the AS group. Significant increase in FosB/ΔFosB-like immunostaining was observed in the dentate gyrus, basolateral amygdala and medial prefrontal cortex of CVS-S rats. Assessments of Neuropeptide Y (NPY), a stress-regulatory transmitter associated with chronic PTSD revealed selective reduction in the hippocampus of CVS-S rats.

Conclusions: Collectively, our data show that cumulative stress potentiates delayed fear memory and impacts defensive responding. Altered neuronal activation in forebrain limbic regions and reduced NPY may contribute to these phenomena. Our preclinical studies support clinical findings reporting worse PTSD outcomes stemming from cumulative traumatization in contrast to acute trauma.
Introduction

Posttraumatic stress disorder (PTSD) is a debilitating, trauma-evoked disorder with high prevalence and morbidity (Kessler et al., 1995). Exposure to intense traumatic events on an acute or chronic scale can lead to PTSD. Studies have demonstrated a steep dose-response curve between trauma frequency and PTSD symptom severity, such that the more traumatic events a person experiences, the greater the intensity of PTSD symptoms (Karam et al., 2014; McTeague et al., 2010). Interestingly, a positive association between the number of combat deployments and risk for PTSD development has also been shown (Reger et al., 2009). Thus, the duration of trauma exposure becomes an important determinant of pathophysiological outcomes. Although this has been observed in clinical settings, there is little evidence on underlying mechanisms or neurochemical contributions to increased pathological reactivity. Animal models can help approach questions raised in clinical research in prospective designs under controllable conditions. In recent years, various preclinical rodent models have been developed for simulation of PTSD-like phenomena (reviewed in Daskalakis et al., 2013, and references therein). These include exposure to single traumatic events such as predator/predator scent exposure (Adamec et al., 2008), shock (Siegmund & Wotjak, 2007), single prolonged stress (Yamamoto et al., 2009), to models simulating chronic trauma, such as predator exposure-social instability (Zoladz et al., 2008), chronic plus acute prolonged stress (Roth et al., 2012), and the chronic variable stress model developed by us (McGuire et al., 2010). There is little information on the comparative effects of cumulative versus acute events on the expression of PTSD-like behaviors. Development of such models would provide information on mechanisms that may promote symptom severity in PTSD stemming from chronic trauma.

In the current study, we tested a paradigm where chronic, unpredictable aversive events were superimposed with a single traumatic event in the form of electric footshocks (chronic variable stress-shock; CVS-S). The comparison group was an acute trauma group which was
exposed only to footshocks without a history of chronic experiences (acute shock, AS). The objective was to simulate comparison of a "combat-like" experience in the CVS-S group, where multiple exposures to unpredictable traumas are superimposed with an index event re-experienced later as traumatic memory with an index trauma alone experience. The CVS-S paradigm was adapted from our previous chronic variable stress (CVS) model expressing enhanced fear recall and reinstatement (McGuire et al., 2010). Choice of footshocks as an index traumatic event was based on rodent PTSD model studies where exposure to single or multiple shocks produces significant effects on conditioned and sensitized responses (Pynoos et al., 1996; Rau et al., 2005; Siegmund & Wotjak, 2007). We measured the effects of these experiences on outcomes relevant to PTSD: associative fear memory responses and acoustic startle as a measure of sensitized response to a non-associative aversive encounter. We analyzed FosB/ΔFosB immunoreactivity to assess regional differences in neuronal activation between cohorts. We also measured peptide transmitter, Neuropeptide Y (NPY) in forebrain areas relevant to PTSD. Accumulating evidence from preclinical and clinical studies supports a role of NPY in stress coping and resiliency (Cohen et al., 2012; Sah & Geraci, 2013; Thorsell et al., 2000; Zhou et al., 2008). Transgenic rats overexpressing NPY in the hippocampus elicit behavioral insensitivity to stress and reduced fear suppression of behavior (Thorsell et al., 2000). Our group (Sah et al., 2009; Sah et al., 2014) has reported significant reduction in central NPY concentrations in individuals with chronic PTSD and its association with intrusive re-experiencing symptoms. In a previous study, we reported enduring changes in forebrain NPY concentrations that temporally correlate with enhanced fear recall and reinstatement in CVS exposed rats (McGuire et al., 2011).

Here, we hypothesized that superimposed multiple adverse experiences would lead to worse behavioral outcomes than a single event, accompanied by adaptive changes in forebrain neuronal activation and reduced NPY peptide concentration.
Materials and Methods

Subjects

A total of 42 male Long-Evans rats (275-300g) were used for all experiments. Animals were purchased from Harlan (Indianapolis, IN) and singly housed in a climate-controlled vivarium on a 12-12 light dark cycle (lights on 6 a.m.). All animals had ad libitum access to food and water except during the brief exposure to stressors. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati, and were conducted during the lights-on period.

Experimental Plan

Figure 1 illustrates the layout of the experimental plan. As shown, there were three experimental cohorts: chronic variable stress-shock (CVS-S), acute shock (AS), and a control reminder shock only group (SC). After a one-week acclimation period, animals were either exposed to a one week, D1-7, CVS exposure (CVS-S) or were handled (AS and SC groups) during the same time CVS-S occurred. On the last day of CVS, footshocks were administered to CVS-S and AS groups. After a one-week recovery period (D14), testing for conditioned fear memory and response to a reminder shock was measured. The SC group was exposed to the context and administered a single shock on D14 to control for the effects of a single shock on acoustic startle response measured on D15. One hour following exposure to startle chamber, brain tissue was collected for neurochemical analysis. Tissue from separate cohorts of CVS, AS and SC was collected either for FosB/ΔFosB immunohistochemistry or NPY ELISA (see below for details).

Chronic variable stress-shock and acute shock paradigms

The chronic variable stress-shock (CVS-S) procedure was a modification of the chronic variable stress procedure described previously by our group (McGuire et al., 2010). We included
an intense traumatic event (footshocks) within this experience for subsequent testing of conditioned fear responses at a delayed time point following recovery. Subjects were randomly assigned to chronic, acute and control groups. Briefly, morning and afternoon stressors for the CVS were administered between 09:00-11:00 and 14:00-16:00, respectively. Stressors were administered in a randomized order with each stressor represented an approximately equivalent number of times, with no stressor administered more than two times to avoid habituation. Stressors included i) cold swim (10m, 16-18°C, in a 41" deep and 55" wide plastic bucket filled to approximately 28" with water), ii) warm swim (20m, 30-32°C), iii) hypoxia (30m, 8%O₂), iv) 1h on an orbital shaker (100rpm, in cage), v) 1h cold room (~ 8-10 °C), vi) 1h restraint (in a well-ventilated Plexiglas tube, small enough to prevent the animals from turning around or moving forwards or backwards) and vii) overnight housing in a small cage (11.5" x 7.5" X 5", standard mouse cage) with no bedding.

On the afternoon of day 7, each rat in the CVS-S and AS groups was placed in the Freeze Monitor apparatus (San Diego Instruments) and acclimated to the chamber for 3 minutes, then received 3 shocks of 1mA intensity, 1s duration administered 1 minute apart. Animals were recorded for post shock freezing for 3 minutes. This represented the primary or index traumatic event that was common to the CVS-S and AS groups. We used a moderate intensity shock paradigm to avoid ceiling effects that would mask potentiation of fear in the CVS superimposed group. Rats were videotaped during and after shocks to compare fear expression during acquisition (training). Enclosures were cleaned between animals with 10% ethanol and shock generating floor bars were then wiped down with a dry paper towel to ensure no moisture remained.
Behavioral testing

Behavioral testing was performed seven days after the termination of CVS-S and AS. This delayed time point is relevant to the enduring symptoms of PTSD and was selected based on previous observation of posttraumatic stress-like outcomes by us and others. All testing was performed in a room separate from the housing area and CVS exposure room.

a) Conditioned fear and reminder shock

To measure conditioned fear on exposure to context rats were placed in the same shock chamber for 5 min. Following this period, a single electric shock (1mA, 1 sec) was administered and the animals stayed in the context for another 5 min. A second shock exposure (in the absence of extinction of previously acquired fear) was performed to simulate trauma in most real life situations, especially combat where re-exposure to a second traumatic event can occur in the absence of recovery from the initial event or exposure therapy. Freezing behavior post initial shock (acquisition), re-exposure to context on day 7 (recall), and post reminder shock exposure was measured. Freezing was defined as the absence of all movement except that necessary for respiration (Fanselow, 1980). Enclosures were cleaned between animals with 10% ethanol.

b) Acoustic startle response

Startle response to an unexpected acoustic stimulus was measured using the SR-LAB startle response system (San Diego Instruments, San Diego, CA). The apparatus included ventilated, soundproof chambers measuring approximately 52cm x 52cm 76cm and contained an enclosure of approximately 12.5cm diameter to keep the animal over the sensor. The enclosure was of sufficient size to restrict but not restrain the animal and allow it to turn around. The chambers were calibrated using the SR-LAB standardization unit, which transmits a precise series of pulses to the sensor located on each enclosure, allowing each chamber to be adjusted to the same read out value for an identical stimulus prior to testing. To further minimize any effect of
measurement differences between the chambers, an equal number of CVS-S, AS and SC
exposed rats were tested in each chamber. The chambers and enclosures were cleaned between
animals with 10% ethanol.

The test consisted of 30 trials. Background noise in the chamber was maintained at 68
dB. The acoustic stimulus was a 40ms, 108 dB burst of white noise emitted at intervals determined
semi-randomly by computer. Inter-stimulus intervals were between 3 and 30 seconds with a
minimum step between interval lengths of 3s. Movement inside the tube was detected by a
piezoelectric accelerometer below the frame. For each trial, measurements were taken at 1ms
intervals for a response window of 150 ms following the startle stimulus using National
Instruments data acquisition software (San Diego Instruments, San Diego, CA). The maximum
voltage change (Vmax) within the recording window over the averaged baseline (5ms) just prior
to stimulus was used for data.

**Immunohistochemistry**

Rats were deeply anesthetized by intraperitoneal injection of Fatal Plus (150mg/Kg,
Vortech, USA) and were perfused transcardially with 4.0% paraformaldehyde in 0.1M
Na₂HPO₄/NaH₂PO₄ buffer, pH 7.5, delivered with a peristaltic pump. Brains were removed and
post-fixed for 24 hours at 4°C. Brains were transferred to 30% sucrose in potassium phosphate
buffered saline (KPBS) until sectioning. Coronal forebrain sections (35 µm) were cut on a sliding
microtome and stored in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1%
polyvinylpyrrolidone, and 30% ethylene glycol) at -20°C until processed for
immunohistochemistry.

Sections were transferred from cryoprotectant to 50 mm potassium phosphate-buffered
saline (KPBS) (pH 7.4) and 0.9% sodium chloride at room temperature (RT). Slices were rinsed
to remove cryoprotectant (5 washes: 5 min) and then incubated in KPBS + 1.0% H₂O₂ for 10 min
at RT. Following this, sections were washed (5 x 5 min) in KPBS at RT and placed in blocking buffer (50 mm KPBS, 10% normal goat serum and 0.2% Triton X-100) for 1 hr. at RT. Sections were incubated overnight in rabbit anti-FosB / ΔFosB primary antibody (H75, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted 1: 300 in blocking in blocking buffer – with 3% NGS instead of 10%). The following morning sections were rinsed in KPBS (5 washes, 5 min) and incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) (1:500 in KPBS + 0.1% bovine serum albumin) for 1 hr. at RT. Sections were rinsed (5 washes, 5 min) in KPBS and then treated with avidin–biotin complex (Vector Laboratories, Inc.) (1:1000 in KPBS + 0.1% bovine serum albumin) for 1 hr. at RT. Following this incubation, sections were rinsed again in KPBS (5 washes, 5 min) and reacted with 0.02% diaminobenzamidine (Sigma Aldrich, St Louis, MO, USA) with 0.05% hydrogen peroxide. Finally, the sections were rinsed in KPB (minus the salt; NaCl), mounted, air dried, then coverslipped in DPX (Sigma Aldrich). Sections were used for quantification of FosB/ΔFosB immunostaining by an experimenter blind to the experimental groups. Slices from areas selected for analysis was based on their relevance to PTSD pathophysiology as well as the observation of FosB/ΔFosB staining: medial prefrontal cortex (mPFC; bregma 3.20mm to 2.20mm), basolateral amygdala (BLA; bregma -2.12mm to -3.3mm) and dorsal hippocampal dentate gyrus (DG; bregma -2.12mm to -3.3mm). It is important to note that the FosB antibody used in our study will recognize both cleaved (ΔFosB) and uncleaved (FosB) forms. Uncleaved FosB diminishes to baseline within 6 h after stimulation of its expression (Nestler, 2001). Due to multiplicity of our paradigm and the timing of perfusion (60 min after termination of behavioral measurements), we are unable to distinguish between the two forms in the current study. We will therefore refer to the immunohistochemical staining as FosB/ΔFosB and use it as a general marker of neuronal activity/plasticity in a particular area. In initial characterization of this antibody in home cage control rats, negligible staining is observed in forebrain limbic areas assessed in our study (data not shown).
The number of FosB/ΔFosB-immunoreactive neurons within the brain regions was quantified with the Scion Image software. Image magnifications were chosen in order to quantify the number of immunoreactive nuclei in one unilateral image from each section. If possible, four images per region, per animal of interest were collected. To quantify the number of immunoreactive nuclei, both a threshold gray level using the density slice option and minimum pixel size were determined using a subset of images for each region with varying signal and background intensities. Using these parameters, the program recorded the number of immunoreactive nuclei within a defined region of interest. The regions were delineated using characteristics of each nucleus taken from the atlas of (Paxinos, 2005), and quantified at a similar distance from bregma within all experimental animals.

NPY ELISA

Separate cohorts of rats were processed for regional NPY-like peptide concentration as described earlier by our group (McGuire et al., 2011). Briefly, the hippocampus, amygdala and prefrontal cortex were dissected from frozen brains from each group by cryostat-sliced sections (see Fig 5 panel for illustration), using bregma −2.12mm to −3.3mm (hippocampus, amygdala) and 3.20mm to 2.20mm (PFC) as stereotaxic coordinates (Paxinos, 2005). Dissected tissue was homogenized in 200–300μl of 0.2 M HCl. The homogenates were boiled for 5 min and cooled on ice. Ten microliter aliquots were removed for later analysis of total protein concentrations. Remaining supernatants were then lyophilized overnight in a Vacufuge TM (Eppendorf, CBRE 3049) to ensure complete drying. Dried extracts were stored at −80°C until ELISA assay. Frozen samples were re-constituted with ELISA buffer and used for NPY ELISA (Peninsula Laboratories, San Carlos, CA, USA). This assay employs antiserum that shows 100% cross-reactivity with NPY (human and rat), and less than 0.02% cross-reactivity to known NPY-related peptides. Authentic NPY standards were used to plot a ten-point standard curve using concentrations ranging from 0-5 ng/ml for quantification of samples. The assay sensitivity was 40 pg/ml with an intra-assay
variation of <6%. Peptide concentration was determined from plotting optical density of unknown samples against the standard curve for NPY. Total protein was determined by Bradford protein assay. Data was calculated for ng NPY/ mg protein.

Data Analysis and Statistical Methods

Behavior was recorded as digitized images using the Freeze Monitor apparatus (San Diego Instruments) and digital camera, and recorded images were played off line for manual scoring by at least two trained individuals blinded to experimental manipulations. Total seconds freezing is expressed as a percentage of exposure time (300s). Data was analyzed using one-way ANOVA or unpaired test as applicable. Startle data is represented as mean +/- SEM of startle amplitude averaged across trials 6-30, analyzed using one-way ANOVA. FosB/ΔFosB and NPY ELISA results are presented as mean +/- SEM and were analyzed using ANOVA. Significance was set at p<0.05. All data was analyzed by using Prism 5, Graphpad, La Jolla, CA.

Results

Potentiated fear acquisition and conditioned responses in rats with prior CVS-S experience

CVS-S elicited significantly elevated fear-related behaviors in comparison to AS. Exposure to fear conditioning on a background of multiple stress experiences in CVS-S rats led to significantly elevated acquisition of fear as assessed by post shock freezing behavior. CVS-S elicited significantly higher freezing (Fig 2A) as compared with the acute shock AS group (mean ± s.e.m., t(26) =3.808; p< 0.0008 vs AS, unpaired t test, n=14/group). After one week of recovery, recall of acquired fear was tested upon re-exposure to context (Fig 2B). Both AS and CVS-S exposed rats showed significantly higher freezing to context as compared to the control (SC) cohort that showed negligible freezing on exposure to context. Importantly, CVS-S elicited
significantly higher freezing as compared to AS. One-way ANOVA revealed a significant effect of stress history as a variable (mean ± s.e.m, $F_{(38)}=58.88$, $p<0.0001$, $n=12-14$/group). Tukey’s post hoc analysis revealed significant differences between AS and CVS-S groups ($p<0.05$) which were both significantly higher than the SC group ($p<0.05$). Exposure to a reminder shock immediately after recall led to significant increases in freezing in CVS-S animals as compared to the AS and SC cohorts (Fig 2C, mean ± s.e.m, $F_{(38)}=60.90$; $p<0.0001$, $n=12-14$/group/one-way ANOVA). Tukey’s post hoc analysis revealed significant difference between AS and CVS-S groups ($p<0.05$) both of which were significantly higher than the SC cohort ($p<0.05$). Furthermore, behavioral responses in the CVS-S versus AS groups immediately following the reminder shock were markedly different. As shown in Fig 2D, AS animals exhibited active coping responses in the form of increased frequency of rearing (looking for escape from the shock box). CVS-S exposed rats on the other hand continued to freeze without active coping responses. Two-way ANOVA revealed a significant effects of stress [$F_{(1,49)} = 4.774; p<0.033; n=14$/group] and testing time [$F_{(1,49)} = 7.78; p<0.0075; n=14$/group], but no stress x time interaction. Bonferroni’s post hoc analysis revealed a significant increase in post shock rearing in the AS group as compared to frequency of rearing in CVS-S animals. No significant difference in rearing frequency between CVS-S and AS groups was observed prior to the reminder shock.

**Altered startle reactivity in rats exposed to CVS-S experience**

In addition to conditioned fear, we also assessed startle response as a measure of sensitized reactivity to an unexpected acoustic stimulus. As shown in Fig 3 mean startle amplitude averaged for trials 6-30 was significantly reduced in CVS-S animals as compared to AS and SC cohorts. One-way ANOVA revealed a significant main effect of stress [$F_{(35)} = 21.42; p<0.0001$, $n=12-14$/group]. Tukey’s post hoc analysis revealed a significant difference between CVS-S and
SC (p<0.05) as well as CVS-S and AS cohorts (p<0.05). No significant differences in startle reactivity was observed between the control SC group and acute shock (AS) group.

**CVS-S exposure results in significant elevation in FosB/ΔFosB-like immunoreactivity (FosB/ΔFosB IR) in forebrain limbic areas regulating stress and fear**

To gain an insight on recruitment of brain regions involved in the control of stress responses in the effects of CVS-S we quantified FosB/ΔFosB IR following startle. As shown in Fig. 4, a significant increase in FosB/ΔFosB IR was observed in CVS-S animals in the dorsal hippocampal dentate gyrus (DG), basolateral amygdala (BLA) and the medial prefrontal cortex (mPFC), while no significant differences were observed between AS and SC cohorts. One-way ANOVA revealed significant main effect of stress in the DG [F(19)=4.381, p<0.0205, n=6-7/group], BLA [F(19)=8.170, p<0.001, n=6-7/group], and mPFC [F(19) =5.76, p<0.010, n=6-7/group]. Post hoc multiple comparison test revealed a significant difference between the CVS-S group and the SC group in the DG (p<0.05). In the BLA and the IL-PFC, CVS-S animals showed significantly higher FosB/ΔFosB IR as compared with AS and SC cohorts (p<0.05).

**Hippocampal Neuropeptide Y (NPY) peptide concentration is selectively reduced in CVS-S exposed animals**

We measured NPY in the hippocampus, amygdala and pre-frontal cortex of CVS-S, AS and SC exposed rats using ELISA. As shown in Fig 5, a selective reduction in hippocampal NPY concentration was observed in the CVS-S cohort as compared to AS, and SC groups. One-way ANOVA revealed a main effect of stress (F(19) = 3.703; p<0.045; n=7/group). Post hoc analysis revealed significantly lower NPY concentration in CVS-S rats versus SC control group (p<0.05).
while no difference was noted between AS and SC cohorts. No significant differences in NPY concentration were observed in the amygdala and the PFC ($F_{(19)} = 0.229; \ p=0.79$, PFC: $F_{(19)} = 1.198; \ p=0.33$, amygdala).

**Discussion**

The current study investigated whether effects of an acute traumatic event on PTSD relevant behavior is potentiated by a history of multiple, adverse experiences prior to the acute event. Our current data show that exposure to chronic variable stress prior to acute stress in the form of footshocks, can potentiate associative fear memory and alter reactivity to non-associative startle stimuli at a delayed post recovery time point. Altered neuronal activity in forebrain stress and fear-regulatory regions and decrement in stress resiliency neuropeptide NPY in the hippocampus may be associated with these effects.

Clinical evidence has shown that exposure to multiple compared with single traumatic events more strongly predisposes towards the development of PTSD. Importantly, symptom severity and chronicity of PTSD appears to be worsened in individuals with multiple traumatic events (Karam et al., 2014; Lang & McTeague, 2011). Subjects who reported previous trauma were significantly more likely to experience PTSD from the index trauma than subjects with no previous exposure to trauma (Breslau et al., 1999). We developed a preclinical paradigm where the impact of previous exposure to multiple, unpredictable stressors on PTSD-relevant responses following an index traumatic event was investigated.

In line with our hypothesis, fear memory related behaviors were significantly worsened by superimposition of multiple stressors with shocks. Our data resonates with previous studies on enhanced fear acquisition, consolidation and impaired extinction in rats following chronic repeated restraint stress exposure (Conrad et al., 1999; Hoffman et al., 2014). In addition, our studies are the first to illustrate that potentiated recall of consolidated fear in rats with previous experience of chronic stress is an effect that persists through post stress recovery. The post recovery time point
is important for dissociation of transient, stress-associated effects from persistent alterations that are temporally relevant to PTSD-like phenomena. We chose a recovery time point when physiological effects of CVS are normalized as previously reported by us and others (McGuire et al., 2010; Ostrander et al., 2006). Prior stress experience can differentially affect the way in which aversive associations are learned and later retrieved. Previous studies have reported facilitation of acquisition and consolidation of fear following stress (Rau & Fanselow, 2009; Shors et al., 1992). Stress evoked alterations in glucocorticoids and synaptic plasticity may have contributed to exaggerated fear responding in the CVS-S group. An interesting finding in our study was the marked difference in coping response to the reminder shock between CVS-S and AS groups. Active coping responses were evident in AS animals while passive defensive responding (persistent freezing) was noted in CVS-S animals following the reminder shock. Collectively, the constellation of behaviors observed in the CVS-S cohort, higher conditioned freezing and reduced post reminder shock rearing activity suggest that the high magnitude of fear may mask active coping responses in this group. Similar passive defensive behaviors were also observed in rats following chronic plus acute prolonged stress, a model of PTSD-like behaviors (Roth et al., 2012).

Contrary to our hypothesis of potentiated acoustic startle response in CVS-S versus AS rats, we observed significantly lower in CVS-S animals as compared to the AS and SC cohorts. Furthermore, we did not observe sensitized startle responses in AS rats as compared to the SC animals. Previous rodent PTSD models using footshock as traumatic event have reported increased acoustic startle behavior (Pynoos et al., 1996; Rau et al., 2005; Servatius et al., 1995). It is possible that the lower shock frequency and intensity in our model did not result in increased startle after 7d recovery. Reduced startle reactivity in CVS-S animals as compared to the other groups is surprising. The acoustic startle response is a fast reflexive response to a strong unexpected acoustic stimulus that has been reported to be enhanced in most rodent PTSD paradigms (Adamec et al., 2008; Cohen & Zohar, 2004; Zoladz et al., 2008), although blunted responses have also been reported (Conti & Printz, 2003). The nature, modality, and timeframe
of stress exposure as well as the time point for startle measurement may lead to these differences.

Previously, a significant decrease in startle responding has been reported following repeated restraint stress (Bijlsma et al., 2010; Conti & Printz, 2003). In agreement with our observation, one study reported blunted startle response at 7 days following termination of stress (Bijlsma et al., 2010). Another possibility may be altered motor activity of CVS-S animals during startle measurement. We have reported enduring increases in motor activity in CVS exposed animals under novel, aversive encounters (McGuire et al., 2010). Previous work has shown that increased activity can inhibit reflexive responses in rats (Wecker & Ison, 1986). Interestingly, blunted startle reactivity was reported in individuals with cumulative traumatization evoked PTSD in comparison with patients with discrete traumatic exposure (Lang & McTeague, 2011; McTeague et al., 2010). Cumulative traumatization by itself may prompt sustained stress and hyperarousal that may ultimately impair defensive physiological reflexes to subsequent stress leading to blunted reflexive responses.

We also observed a significant decrement in peptide stress resiliency transmitter NPY in the hippocampus of CVS-S animals. In contrast to our previous study (McGuire et al., 2011), these changes were selective to the hippocampus and not observed in the amygdala or the PFC. It is possible that exposure to shocks in conjunction with CVS provided a strong contextual adverse experience that may have influenced NPY in the hippocampus, a prime area for processing context-associated memories. Increased hippocampal NPY was reported in rats after two weeks of chronic unpredictable stress (Hawley & Leasure, 2012). Stress duration, modality and importantly, post stress recovery (early versus delayed) may contribute to differential effects on NPY expression. Our data are in sync with observations of reduced hippocampal NPY in the predator stress and the single prolonged stress (SPS) models of PTSD (Cohen et al., 2012; Serova et al., 2013). Importantly, in both these studies NPY quantification was also performed 7d post stress exposure as in the current study. Hippocampal NPY content associates with improved coping and adaptive behaviors in rats (Bardi et al., 2012). Intra-hippocampal administration of
NPY attenuates predator-cue associated freezing behavior (Cohen et al., 2012). Furthermore, transgenic overexpression of NPY in the hippocampus results in absent fear suppression of behavior in a punished drinking test (Thorsell et al., 2000). Collectively, all evidence suggests that an enduring decrease in NPY in the hippocampus of CVS-S animals may contribute to potentiated fear and reduced coping behaviors observed in this cohort.

Prior exposure to repeated stress can lead to changes in neural circuits that facilitate the mnemonic encoding of subsequent experiences of the stress. Behavioral effects are accompanied by the reorganization of neural structures, characterized by changes in synaptology and dendritic morphology, likely mediated by chronically driven stress-sensitive circuits (Carvalho-Netto et al., 2011; Cook & Wellman, 2004; Vyas et al., 2002). It is well established that fear regulatory regions such as the hippocampus, prefrontal cortex and the amygdala are particularly sensitive to prolonged stress (Flak et al., 2012; Ghosh et al., 2013). Significantly increased FosB/ΔFosB labeling was observed in the basolateral amygdala, hippocampal dentate and infralimbic prefrontal cortex in CVS-S exposed animals as compared with AS and SC exposed animals. This suggests that the chronicity of stress altered the recruitment of these nuclei within primary brain circuits regulating emotional responses. Significant effects of chronic stress exposure have been reported in the hippocampus and basolateral amygdala (Magariños & McEwen, 1995; Vyas et al., 2002). Consistent with our results significant neuronal activation within the BLA and hippocampal subnuclei was reported in previous models of chronic stress evoked fear as well as footshock induced associative contextual fear in an animal model of PTSD (Hoffman et al., 2014; Sauerhöfer et al., 2012). Stress modulatory lasting effects on fear are contributed to by increased neuronal excitability and plasticity at excitatory synapses in the basolateral amygdala (Roozendaal et al., 2009), a primary site for acquisition and consolidation of fear. Under conditions of repeated stress, the balance between synaptic excitation and inhibition may lead to changes that are more persistent in synaptic plasticity (Roozendaal et al., 2009). Association of the medial prefrontal cortex to chronic stress evoked potentiation of fear
responses has been reported (Wilber et al., 2011). The prefrontal cortex is part of a circuit whose coordinated activation with the basolateral amygdala and hippocampus is critical for processing of fear memory. The lack of differences between AS and SC groups suggest that the acute shock exposure in itself may not be robust enough to produce effects on neuronal activation/plasticity. Collectively, our data implies that altered activity in prime forebrain limbic areas regulating stress and emotion may contribute to differences in behavioral responses between CVS-S, AS and SC exposed rats.

**Conclusions**

The ‘over-acquisition’ and expression of fear memories is thought to contribute to the genesis of trauma-related disorders such as PTSD and may perpetuate distress after the trauma: humans with PTSD have extremely strong memories of the PTSD-inducing trauma. However, populations with repeated trauma exposure or greater life stress accumulation demonstrate higher risk for the development of PTSD and worse symptom severity. We reported reduced CNS concentrations of resiliency hormone NPY in combat veterans with chronic PTSD (Sah et al., 2009). Results of the current study provide evidence of worsened associative fear, impaired coping and altered startle reactivity in rats with superimposed chronic variable stress in comparison to an acute stress insult alone. It is not evident from this study whether these effects are due to CVS exposure alone or CVS superimposed with shock, since a CVS alone group was not included. However, previously we observed no effects of CVS alone on acoustic startle at 7d post recovery (McGuire and Sah, unpublished observations), suggesting that a combined CVS and shock insult may be responsible for altered behaviors. Changes in activity within limbic forebrain areas regulating stress and emotion, as well as enduring reduction in NPY may contribute to these outcomes. Our preclinical model corroborates clinical findings that report
increased symptom severity and compromised defensive responding in PTSD stemming from cumulative traumatization in contrast to single trauma exposure.

Fig. 1

**Fig. 1:** Schematic representation of experimental layout and groups. Following acclimation, rats were assigned to CVS-S, AS and SC cohorts (n = 14/group). CVS-S rats were exposed to variable stressors for 7 days (D1–7) while AS and SC cohorts were handled. On the final day of CVS, rats in the CVS-S and AS groups were administered three electric shocks (1 mA, 1 s). Rats recovered for 7 days. Behavioral testing for conditioned fear and response to a single reminder shock was performed on D14. On D15 all groups were assessed for acoustic startle as a measure for sensitized behavioral response to an acute auditory stimulus. Brains were collected for FosB/ΔFosB-like immunoreactivity and neuropeptide Y (NPY) ELISA (CVS-S = chronic variable stress-shock, AS = acute shock, SC = shock control).
Fig. 2: CVS-S potentiated acquisition of fear, contextual fear recall and post reminder shock shock responses. (A) Freezing behavior on D7 of AS and CVS-S rats following initial conditioning shocks. (B) Contextual conditioned fear on D14 in AS and CVS-S rats on context exposure following 7 d recovery period in comparison with the SC group. (C) Freezing behavior on D14 following single reminder shock in AS, CVS-S and SC rats. CVS-S rats showed significantly greater freezing as compared to AS-exposed rats, while both groups froze more than the SC group. (D) Rearing frequency on D14 of AS and CVS-S rats prior to (pre) and following (post) the reminder shock. AS-exposed rats showed significantly increased rearing post reminder shock. Data are shown as mean ± SEM; n = 12–14 rats per group. *p < 0.05 significance between AS and CVS-S groups; **p < 0.05 significance between AS, CVS-S versus SC (A: unpaired t-test, B, C: one-way ANOVA and Tukey's post-hoc, D: two-way ANOVA and Bonferroni's post-hoc). CVS-S = chronic variable stress-shock, AS = acute shock, SC = shock control.
Fig. 3: CVS-S exposure evokes reduced acoustic startle response during recovery. Mean startle amplitude in CVS-S, AS and SC groups averaged over 25 trials. Data are shown as mean ± SEM (n = 12–14 rats per group). *p < 0.05 versus SC and AS groups (one-way ANOVA and Tukey’s post-hoc). CVS-S = chronic variable stress-shock, AS = acute shock, SC = shock control.
Fig. 4: FosB/ΔFosB-like immunoreactivity in the hippocampal dentate gyrus (DG), basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) of CVS-S, AS and SC rats. Left panels show representative images of dark-field photomicrographs (immunoreactive nuclei are white) from DG (A–C), BLA (D–F) and mPFC (G–I) of SC, AS and CVS-S rats. Right panels show bar graphs of quantified data. Significant increase in FosB/ΔFosB immunoreactive cells was observed in CVS-S-exposed rats in the DG (*p < 0.05 versus SC), BLA (*p < 0.05 versus SC and AS) and mPFC (*p < 0.05 versus SC and AS), (one-way ANOVA and Tukey's post-hoc). Data are shown as mean ± SEM, n = 6–7/group. Bottom panels show stereotaxic illustrations adapted from the atlas of Paxinos & Watson. (J) Sections were quantified from the DG and BLA as shown with dotted lines from slices using bregma (Br) −2.12 mm to −3.30 mm. (K) Sections for the mPFC were quantified as shown with dotted lines from Br 3.2 mm to 2.20 mm. (CVS = chronic variable stress-shock, AS = acute shock, SC = shock control). Scale bar = 5 µm (panel I).
Fig. 5: Neuropeptide Y (NPY) concentration in forebrain limbic brain regions measured by ELISA. Panels on the left show stereotaxic illustrations adapted from the atlas of Paxinos & Watson. (A) Tissue from the hippocampus and amygdala was collected as shown with dotted lines from slices using bregma (Br) −2.12 to −3.30 (B) Tissue for the medial prefrontal cortex was collected as shown with dotted lines from Br 3.2 to 2.20. Bar graphs show NPY ELISA data from SC, AS and CVS-S rats. ANOVA and Tukey's post-hoc: Significant reduction in NPY concentration was observed in the hippocampus from CVS-S-exposed rats (*p < 0.05 versus SC group; one-way ANOVA and Tukey's post-hoc). No significant changes in NPY in the amygdala and medial prefrontal cortex were observed between SC, AS and CVS-S cohorts (p > 0.05). Data are shown as mean ± SEM, n = 7/group. (CVS = chronic variable stress-shock, AS = acute shock, SC = shock control).
Chapter 5
General Discussion and Future Directions
Summary of Dissertation

This dissertation investigated the role of forebrain NPY, specifically in the infralimbic prefrontal cortex (IL-PFC) and the amygdala in the regulation of PTSD-relevant behaviors. Regulation of fear memory related behavior was a primary focus of our studies.

Higher prefrontal NPY expression is observed in individuals carrying the rs16147 T>C polymorphism in the NPY gene, which is associated with negative affect in high adversity (Sommer et al., 2010). Given no prior studies exist on the functional role of NPY in the prefrontal cortex an area central to PTSD pathophysiology, studies were undertaken under chapter 2, to investigate regulation of behaviors associated with fear, depression and cognitive tasks associated with working memory. Neuroendocrine response to restraint stress was also assessed. In another study within this section, retrograde tracing studies were performed to identify intrinsic and extrinsic NPY innervation of the PFC. In Study 1, NPY was administered into the IL-PFC in rats exposed to a cued fear conditioning paradigm and effects on IL-regulated extinction and retrieval of extinction were assessed. NPY infusion into the IL-PFC prior to extinction training led to a dose-dependent impairment of extinction retrieval tested 24 hr. later. Post extinction treatment confirmed that NPY infusion compromised the consolidation of extinction learning. We further report that NPY- Y1 receptor (Y1R) in the IL mediate NPY effects on extinction retrieval. Contributions of IL-NPY to other mPFC-regulated behaviors and HPA response were investigated. No effects of IL NPY infusion on corticosterone response to restraint stress, immobility in the forced swim test, and working memory in the delayed win-shift were observed. To further characterize the functional effects of NPY in the IL, electrophysiological effects of NPY on IL layer V pyramidal neurons were measured. NPY significantly increased eIPSCs and showed a modest reduction of eEPSCs, suggesting that NPY can regulate the balance between synaptic excitation and inhibition onto layer V pyramidal neurons in a way that may reduce the firing rate of the output neurons of the IL-PFC. In support
of this finding, colocalization of Y1Rs with CaMKII (a marker or projection neurons) was observed. Figure 1 shows a theoretical circuit showing how NPY may be effecting behavioral outcomes via the IL-PFC. In individuals expressing rs14167 gene polymorphism or with a history of chronic stress exposure, elevated prefrontal NPY may contribute to IL hypoactivity, resulting in impaired extinction memory and increased susceptibility to anxiety disorders such as PTSD. Findings from this section have been published (Vollmer et al., 2016).

In Study 2 of chapter 2, we investigated the origin of NPY projections to the IL-PFC. Our observations in study 1 revealed functional contributes of NPY in the IL-PFC. Since the majority of the NPY-like immunoreactivity in the IL-PFC is localized to fibers, it is relevant to identify areas with NPY efferents to the IL to gauge regulatory influences on IL-NPY function. Using the retrograde tracer FluoroGold (FG) and NPY02, an antibody that preferentially labels NPY expressing cell bodies; we identified a primary NPY projection to the IL from the caudal dorsal raphe nucleus, dorsal subdivision. Since both the IL (McKlveen et al., 2013; Rive et al., 2013) and dorsal raphe (Heller, 2016; Michelsen et al., 2007) play important roles in the regulation of stress related behavioral responses, NPY within DR-IL circuitry may constitute an important regulatory system modulating the effects of stress-associated fear memory of relevance to disorders such as PTSD. Figure 2 shows a theoretical circuit indicating how NPY projections from the DRN to the IL-PFC may play a role in response to a stressor. Other areas that contribute to NPY efferents to the IL, but to a lesser degree, were the ventral hippocampal CA1, and area regulating contextual fear as well as neurons within the IL and PL suggesting intrinsic NPY circuits within the mPFC. More in depth studies in future are needed to fully elucidate the role of the raphe-IL NPY circuit in stress and emotional responses.

Chapter 3 focused on the contributions of NPY in the amygdala on PTSD-relevant behaviors, primarily regulation of fear memory, using pharmacological and viral overexpression approaches. In Study 1, NPY infusion in the BLA in fear conditioned rats prior to extinction
training was found to significantly decrease conditioned freezing to tone, as well as facilitate extinction. Given the limitations of acute NPY treatments for chronic stress studies and the confounding effects of chronic NPY infusion on the downregulation of Y receptors, we employed lentiviral approaches in Study 2. Behavioral studies were conducted with NPY over expression vector, CMV-NPY. In experiment 1 of Study 2, CMV-NPY evoked NPY overexpression in the BLA had no significant effect on anxiety-like behavior or acoustic startle response, suggesting that NPY in the BLA does not appear to regulate anxiety/startle under no stress conditions. In experiment 2 of Study 2, delivery of NPY lentivirus and its expression in intercalated cell (ITC) enriched capsular areas surrounding the BLA significantly increased conditioned fear and impaired extinction. This effect was potentiated in animals exposed to chronic variable stress (CVS). Although unanticipated, our results provide the first evidence of fear regulatory effects of NPY in the lateral ITCs, which are enriched in inhibitory GABAergic interneurons. A small subset of animals that received NPY in the BLA and CeA showed reduced conditioned freezing in agreement with previous studies reporting fear-reducing effects of NPY in the amygdala. To date, functional effects of NPY in the amygdala have focused on excitatory pyramidal projection neurons that are inhibited by NPY, however, regulation of inhibitory mechanisms by NPY has not been studied. Recent studies have shown the relevance of inhibitory mechanisms, particularly the medial and lateral ITC cell clusters in gating cortical input to the CeA and BLA, respectively (Ciocchi et al., 2010; Ehrlich et al., 2009; Haubensak et al., 2010; Denis Paré et al., 2004). Elevated NPY in these areas may limit GABAergic inhibition that will result in disinhibition of projection neurons within output nuclei leading to enhanced fear (Fig. 3B illustrates these effects). Our IITC findings should be verified using pharmacological agonist-antagonist studies. This could be done using agonist and antagonists targeting different Y receptors, due to the high level of Y2 in the ITC cells compared to the high level of Y1 in the BLA. NPY and NPY-Y2 receptors (presynaptic autoreceptors) have been localized to ITC clusters (Wood et al., 2015) therefore their role in modulation of GABA release and effects on downstream BLA circuits
should be investigated. These results suggest potential regulation of cortical-amygdala fear circuits by NPY in paracapsular areas. Our results also highlight that fear-regulatory effects of NPY in the amygdala are complicated and highly site-specific.

In chapter 4, we investigated NPY in a rodent paradigm that simulated and compared the effects of an acute traumatic event with the same event occurring on a background of chronic variable stress (CVS). This is an important distinction for PTSD research, as those who experience trauma on a chronic scale tend to have worse outcomes than those who experience a singular traumatic event (Karam et al., 2014; McTeague et al., 2010). As hypothesized, rats who experienced the “traumatic event” (simulated by footshocks) on a background of CVS (CVS-S) had increased fear acquisition and conditioning compared to both the acute shock (AS) and control (SC) groups. Interestingly, we also observed differing behavioral “coping” responses between the CVS-S and AS group. While the CVS-S animals spent most of their time freezing following the reminder shock, the AS group displayed “active coping” by showing increased frequency of rearing. These passive coping responses have been observed in other models of PTSD-like behaviors such as the chronic plus acute prolonged stress model (Roth et al., 2012), and may provide insight into the differences in outcomes that patients with PTSD stemming from chronic traumatization experience. Surprisingly, contrary to our hypothesis, CVS-S rats showed reduced acoustic startle response (ASR). This could be due to increased motor activity seen in CVS exposed animal (McGuire et al., 2010) inhibiting reflexive responses (Wecker & Ison, 1986) such as startle. Interestingly, patients with cumulative traumatization related PTSD show blunted startle reactivity compared to single-trauma PTSD patients (McTeague et al., 2010). To investigate potential association of NPY in this paradigm, we examined forebrain NPY in the amygdala, PFC and hippocampus using ELISA. Although no significant changes were observed in the amygdala and the PFC, significantly reduced NPY concentration was observed in the hippocampus. This may potentially result from the recruitment of this area in the heavily
contextual paradigm that was used in our studies. Other rodent models of PTSD such as single prolonged stress (SPS) and predator exposure have also reported reductions in hippocampal NPY (Cohen et al., 2012; Serova et al., 2013). Examination of FosB/ΔFosB reactivity in the basolateral amygdala, hippocampal dentate and infralimbic prefrontal cortex revealed significantly increased immunoreactivity in the chronically stressed CVS-S group, implicating these brain areas in the observed behavioral differences.

**Future Directions**

Assessment of NPY in forebrain areas relevant to PTSD revealed important information, although questions remain regarding the role of the NPY system in the regulation of PTSD-relevant behaviors. The most important conclusion that was common to most studies conducted in this dissertation was the site-specificity of NPY effects. Many results, especially the IL-PFC and the lateral ITC studies revealed novel, unanticipated findings that challenged previous concepts supporting NPY as a fear-reducing peptide. However, future investigation on this system is needed to fully understand contributions of NPY in a circuit-specific manner using sophisticated tools such as optogenetics and DREADDs.

One of the main novel findings of this dissertation was IL-PFC NPY mediated regulation of extinction memory, an outcome of relevance to PTSD. In addition, functional regulation of excitatory/inhibitory neurotransmission and neuroanatomical characterization of IL-NPY at a cellular and circuit level was also achieved. These effects are contrary to well-established fear-reducing effects of amygdalar and hippocampal NPY. The DRN-IL NPY circuit identified in our studies is of interest to PTSD. DRN-IL reciprocal connections are implicated in regulating uncontrollable stress (Grahn et al., 1999). Given the sensitivity of PFC NPY to chronic stress (McGuire et al., 2011) and relevance of DR-IL circuits in the regulation of controlled versus uncontrollable stress this circuit is particularly relevant for PTSD pathophysiology. As a next
step, identification of neurons within the IL-PFC that are targeted by NPY neurons in the DRN and their effects on stress and fear outcomes would be relevant.

In chapter 3, the role of NPY in the amygdala was investigated. We found opposing effects of NPY on fear regulation dependent on the targeted site: while BLA NPY infusion resulted in reduced fear, increased expression within the lateral ITC cluster had fear enhancing effects. While the role of NPY in the BLA nucleus has been studied extensively in relation to anxiety and stress, only recently have researchers focused on NPY within the ITC clusters, particularly the lateral ITC cluster. A recent paper reported high expression of NPY within paracapsular ITC cells and showed co-localization of NPY and the D1 dopamine receptor within ITC neurons (Wood et al., 2015). Interestingly, dopamine inhibits lateral ITC interneurons that results in disinhibition of BLA projection neurons (Marowsky et al., 2005). Since information from lentiviral studies has limitations due to non-specific expression in non-NPY neurons, direct pharmacological intervention is necessary to verify NPY-ITC behavioral data. Electrophysiological studies examining NPY effects on IPSCs generated by these cells as well as action potentials generated by BLA pyramidal cells would be crucial to further understand NPY fear modulation via this circuit.

In chapter 4, we explored the differences between a singular traumatic experience and that same experience with a background of chronic stress. It well known in the field of PTSD research that chronic traumatization PTSD patients tend to have worse outcomes (Karam et al., 2014; McTeague et al., 2010). However, the neurobiological differences between these patients and patients that experience a single trauma is not yet known. Our results point to increased activation following a stressor in the PFC, amygdala and hippocampus, a specific NPY deficit in the hippocampus, and differences in coping responses to trauma reminders. As this study was conducted first, we were unaware at the time of the possible contribution of the NPY projection from the DRN to the IL-PFC. It would be interesting to compare neuronal activation in DRN NPY...
projection neurons in CVS-S vs. AS and SC animals. This would give us more information on the contribution of this circuit and whether it is recruited more or less in chronic vs. acute trauma. Recent studies in a predator stress PTSD model have reported reduced contextual fear memory in animals treated with NPY in the hippocampus (Cohen et al., 2012). While targeting hippocampal subfields is complicated, future studies are required to tease out contributions of hippocampal NPY to behavior.

**Therapeutic Implications**

There is a reasonable amount of evidence from ‘bench and bedside’ observations supporting NPY and NPY receptors as potential therapeutic targets for PTSD (reviewed in Brothers & Wahlestedt, 2010; Sabban et al, 2015). Targeting the NPY system to enhance NPY function in vulnerable individuals prior to predictable trauma (e.g. combat exposure) may facilitate resiliency and reduce the likelihood of developing PTSD. Preclinical evidence suggests that central NPY supplementation following traumatic stress can prevent the development of PTSD-like behaviors. Most data indicate that an increased availability of CNS NPY may be beneficial for several physiological responses relevant to PTSD. In this regard, administration of NPY peptide /Y1 agonists /Y2 antagonists may be effective treatments. In mice, NPY administered intravenously can enter the brain by diffusion across the blood brain barrier (Kastin & Akerstrom, 1999), although NPY distribution and dynamics in humans is less well understood. Use of blood-brain barrier penetrant non-peptide NPY Y2 receptor antagonists is an interesting option (see Saldanha et al., 2010 for review). Several potent Y2 antagonists with desirable brain penetrant properties have been synthesized (Brothers & Wahlestedt, 2010; Mittapalli et al., 2012; Shoblock et al., 2010), although preclinical studies have been limited. Compound JNJ-31020028 elicited anxiolytic and antidepressant effects in rodent models (Cippitelli et al., 2011; Morales-Medina et al., 2012). This therapeutic approach has limitations: while Y2 antagonists may promote anxiolysis, they are likely to have adverse effects on the processing of fear.
memories, given recent evidence showing reduced fear expression via Y2 receptors (Verma et al., 2015). Moreover, systemic administration of NPY modulators may have undesirable side effects, given the modulatory effects of NPY on vasoconstriction, inflammation, angiogenesis and adipogenesis (Hirsch & Zukowska, 2012). Intranasal (IN) NPY delivery is an interesting option, as it has the potential to increase peptide concentration directly in the CNS without significant effects in the periphery. This approach has worked successfully in the SPS and PSS rodent models of PTSD (Cohen et al., 2015; Serova et al., 2013). Investigators at Mount Sinai are using this approach to study whether effective concentrations of NPY can be delivered to the human CNS (Clinical trial NCT00748956 and ongoing studies). While the IN route has worked in rodents there are several unknowns to consider before translation to humans. Differences in blood brain barrier architecture, permeability, peptide uptake and degradation mechanisms between rodents and humans are important in assessing how much peptide needs to be delivered via the IN route to reach effective CNS concentrations. Thus, translation of NPY therapeutics from preclinical studies to humans has limitations and challenges despite the efficacy of NPY in rodent models. It would also be important to consider regional disparity in beneficial versus adverse effects of NPY on PTSD-relevant behaviors (as demonstrated by this dissertation). This is especially relevant for the regulation of fear conditioning, as we have seen differential effects of NPY in the amygdala versus PFC, and others have reported differences with NPY in the hippocampus as well.

Conclusions

Figure 4 illustrates a potential link between NPY dysregulation and PTSD. A combination of genetic and environmental factors, such as early adversity or chronic stress exposure, may result in maladaptive functioning of the NPY system. This may promote anxiety, hyperarousal or impaired processing of fear memories, increasing a risk to develop PTSD. Although studies support a potential relevance of NPY in PTSD, there are several knowledge gaps in our
understanding of how NPY may associate with posttraumatic physiology. So far, research groups have taken a highly fragmented view of the NPY system focusing on specific brain areas and behaviors. It would be useful to employ an interconnected, circuit level approach to start investigating how NPY and NPY receptors in different brain regions regulate circuits and transmitters impacting PTSD-relevant outcomes. For example, there is evidence of a dysfunctional connectivity and crosstalk between the amygdala and PFC in PTSD. Gaining insights on how NPY regulates PFC/amygdala circuits and its impact on behavioral outcomes (primarily fear) will be useful. Currently, information on CNS NPY in PTSD has been restricted to analysis in the CSF. While providing valuable information, NPY concentration in discrete brain areas remain unknown. Since the uptake and dynamics of NPY across CSF and regional brain compartments is unclear, we cannot assume a global reduction of NPY in the brain based on CSF analysis. Postmortem studies in healthy versus PTSD subjects are warranted in this regard. Based on evidence suggesting an interaction between early adversity and NPY gene polymorphisms, it would be important to investigate epigenetic modifications in segments around SNPs identified in NPY and NPY receptor genes in association with PTSD diagnosis. These studies may potentially lead to the development of biomarkers for PTSD risk and susceptibility. In conclusion, the NPY system is of relevance to PTSD pathophysiology. However, the applicability of NPY and NPY receptors in the development, diagnosis and treatment of PTSD still remains to be demonstrated.
Fig. 1: Regulation of fear memory by infralimbic NPY: potential mechanisms (A) Normal function of the IL PFC, activating inhibitory ITC cells gating the CeA, and reducing CeA output. (B) Effect of NPY on the IL PFC and downstream circuits and fear behavior output.
Figure 2: Speculative role of the DRN to IL-PFC NPY projection. NPY projections from the DRN may inhibit PFC projection neurons, leading to decreased excitation of the medial ITC cells and increased CeA output, leading to increased fear responses.
Figure 3: Effects of NPY supplementation in different amygdala subregions. A, NPY supplementation in the BLA reduces fear expression. B, NPY supplementation in the lateral ITC cluster increases fear expression, possibly through reduced inhibition of the BLA. BLA, basolateral amygdala; ITC, intercalated cells; M-ITC, medial intercalated cell cluster; L-ITC, lateral intercalated cell cluster.
Figure 4: Potential links of NPY with PTSD susceptibility. A combination of genetic and environmental factors such as early adversity or chronic stress exposure may result in maladaptive functioning of the NPY system. This may promote impaired processing of fear memories promoting the development of PTSD.
Bibliography


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