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The External Globus Pallidus: Bidirectional Control Over Anxiety-Related Behavior Mediated by CRFR1

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by

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A

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by

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Dedication

I would like to dedicate this dissertation to my mother, Moira E. Mañon, and my grandmother (Mimi), Patricia E. Lajeunesse.

These two women have supported me through every event in my life. Together, they raised me and taught me that I have the ability to do anything. It is because of them, and the wish to make them proud, that I decided to continue my studies and obtain my PhD.

Without them, I would never have reached this goal. I have had many struggles in the past where I have fallen, and they have always been there to pick me up. Their love and support keeps me going when times are rough, and their laughter makes me smile when times are great.

I respect and love these women with all of my heart, and I am so glad that I have the opportunity to dedicate this body of work to them. They truly deserve it.

Acknowledgements

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I would like to acknowledge the terrific staff at GSBS who support the students as if they were their own children. Without the staff, my time at GSBS would have definitely not have been as enjoyable.

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to acknowledge my girls: Ashley Stella, Mary (Katie) Rountree, Kendra Smith-Phillips, Sonya Garza, and Andrea Gallagher for being the most supportive and loving group of people. Both the guys and the girls are truly my second, chosen family.

Especially, I want to acknowledge the GSBS entering class of 2013. We made it through two weeks of “boot camp” which was not the most fun of times. However, all of the time spent together lead to strong and lasting friendships that I will cherish for the rest of my life.

Lastly, I want to acknowledge my wonderful husband Diego Anibal Morales-Scheihing. His unconditional love and support keep me going and make everyday full of laughter.

Abstract

THE EXTERNAL GLOBUS PALLIDUS: BIDIRECTIONAL CONTROL OVER ANXIETY-RELATED BEHAVIOR MEDIATED BY CRFR1

Albert Lee Joseph Hunt, Jr., B.S.

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Corticotropin-releasing factor receptor 1 (CRFR1), the principle receptor responsible for the anxiogenic activity of the stress peptide CRF, is abundantly expressed in the external globus pallidus (GPe) raising the question whether activity in the GPe is altered in response to stress. I show that CRFR1 expressing neurons are of the "prototypic" subtype of GPe neurons. I provide evidence of novel circuits from CRF neurons in stress-responsive nuclei, including the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (CeA), that provide excitatory input to the GPe. Additionally, I show that activation of CRFR1 neurons using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) increases anxiety-related behavior and movement. I show that anxiety-related behavior and movement are decreased in response to activation of Npas1+ neurons, a class of neuron in the GPe that are primarily of the "arkypallidal" subtype. My evidence indicates that CRF neurons may project to the GPe to modulate anxiety-related behavior and movement through differential synaptic input to distinct GPe neuronal subtypes. CRF to GPe circuits provide possible therapeutic avenues to treat anxiety disorders comorbid with basal ganglia neurodegenerative diseases that cause aberrant activity in the GPe such as Parkinson's disease.

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Abbreviations

6-OHDA	6-hydroxydopamine
ACTH	adrenocorticotropic hormone
AVP	arginine-vasopressin
BAC	bacterial artificial chromosome
BG	basal ganglia
BST	bed nucleus of stria terminalis
BSTld	lateral dorsal subdivision of the bed nucleus of stria terminalis
CAS	central autonomic system
CeA	central nucleus of the amygdala
CeAl	lateral central nucleus of the amygdala
CeAm	medial central nucleus of the amygdala
ChR2	channelrhodopsin-2
CNO	clozapine N-oxide
CNS	central nervous system
Cort	cortisol/corticosterone
CRF	Corticotropin-releasing hormone
CRF-BP	Corticotropin-releasing hormone binding protein
CRFR1	Corticotropin-releasing hormone receptor 1
CRFR2	Corticotropin-releasing hormone receptor 2
CSF	cerebrospinal fluid
D1	dopamine receptor 1

D2	dopamine receptor 2
DREADD	designer receptor exclusively activated by designer drugs
DST	dexamethasone suppression test
EP	entopeduncular nucleus
EPM	elevated-plus maze
ER	endoplasmic reticulum
GP	globus pallidus
GPe	external globus pallidus
GPi	internal globus pallidus
GR	glucocorticoid receptor
HPA-axis	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventricular
IP	intraperitoneal
INTs	interneurons
KO	knock out
LC	locus ceruleus
LDT	light dark transfer
LPS	lipopolysaccharide
MDD	major depressive disorder
MR	mineralcorticoid receptor
MSNs	medium spiny neurons
NTS	nucleus of the solitary tract

OFA	open field assay
PBl	lateral parabrachial nuclei
PD	Parkinson's disease
PFC	prefrontal cortex
PLC	phospholipase C
PPE	preproenkephalin
PTRV	pseudo-typed rabies virus (technique)
PTSD	posttraumatic stress disorder
PV	parvalbumin
PVN	paraventricular nucleus of the hypothalamus
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SPNs	spiny projection neurons
STN	subthalamic nucleus
STR	striatum
UCN1	urocortin 1
UCN2	urocortin 2
UCN3	urocortin 3
VLM	ventrolateral medulla
WT	wild type

Introduction

The Stress Response, HPA-axis, and Corticotropin-releasing factor

Hans Selye is considered the father of stress research. After many years of experiments, he defined stress as the nonspecific response of the body to any demand for change or challenge to homeostasis. In 1936, Selye was the first to provide evidence of the physical effects of stress on the body. In what he termed “a typical syndrome,” he described three stages that occurred independent of what the “damaging agent” or stressor was. The first stage he termed the “Alarm Reaction.” Today, we would call this the activation of the HPA-axis which will be discussed in the following sections. The second stage, the “Stage of Resistance.” In this stage, the body attempts to counteract the changes that the stressful stimulus produced. Lastly, he termed the third stage, the “Stage of Exhaustion.” In this stage, the body has depleted all of its resources needed to repair itself back to a state of homeostasis. He believed that all three stages of this “syndrome” represented a collective effort of an organism to adapt to new conditions and coined this syndrome the “general adaptation syndrome.” Hans Selye’s paper “A syndrome produced by diverse nocuous agents” continues to serve as the foundation for future stress research (Selye 1936; Holsboer and Ising, 2010; Selye, 1950).

Regulation of homeostasis in the presence of a stressor (lack of sleep, cell death, threat to life, working out; any threat to homeostasis) requires activation and initiation of multiple systems including the endocrine, nervous, and immune systems. The activation of these systems is known as the stress response (Carrasco and Van de Kar, 2003; Chrousos, 1992a). As a result of activation of the stress response, behavioral and physiological adaptations that increase an organism's chances for

survival when faced with a challenge to homeostasis are enacted through the sympathetic nervous system. Physiological changes initiated by the stress response include increased cardiovascular tone and respiratory rate. Also, the stress response suppresses activities in the body including inhibition of feeding, digestion, growth, and immune system functioning (Habib et al., 2001; Sapolsky, 2000). Some of the behavioral changes that occur when faced with a threat are increased arousal, improved cognition, and a reduction in the sensation of pain (Charmandari et al., 2005; Chrousos, 1992a). The main structures of the stress response are located in the paraventricular nucleus of the hypothalamus (PVN), the anterior lobe of the pituitary gland, and the adrenal gland. Collectively, these structures form the hypothalamic-pituitary-adrenal (HPA)-axis. Additional neural structures involved in the stress response include the locus ceruleus (LC)-noradrenergic system, sympathetic adrenomedullary circuits, and parasympathetic systems (Chrousos, 1992b; Tsigos and Chrousos, 2002; Whitnall, 1993). The HPA-axis, in combination with the efferent sympathetic/adrenomedullary system, represent the effector limbs by which the brain influences all body organs during exposure to a noxious stimuli (Gold et al., 1988).

Corticotropin-releasing factor (CRF), also known as Corticotropin-releasing hormone (CRH), is a 41 amino acid peptide originally isolated from ovine hypothalamic extracts (Vale et al., 1981). Neurons located in the medial parvocellular subdivision of the PVN release CRF, the primary regulator of the HPA-axis, which travels through the hypophyseal portal to bind corticotrophs and stimulate the secretion of corticotropin, now known as adrenocorticotropic hormone (ACTH) from the anterior pituitary (Rivier and Vale, 1983; Vale et al., 1981). In fact, Selye predicted the

existence of an “X factor” that performed this function some 30 years before the discovery of CRF (Selye, 1950). Arginine-Vasopressin (AVP) released by AVP PVN neurons acts synergistically with CRF to amplify ACTH secretion, however AVP release does not result in ACTH secretion alone. Additionally, there is reciprocal positive interaction between CRF and AVP in the hypothalamus with each neuropeptide stimulating the secretion of the other (Smith and Vale, 2006; Tsigos and Chrousos, 2002). ACTH travels through the systemic circulation to the adrenal cortex and stimulates the synthesis and release of glucocorticoids, namely cortisol in humans or corticosterone (Cort) in mouse (Fig.1). Glucocorticoids act as the downstream regulators of the HPA-axis and act through two receptors; mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). The main effects of the stress response are mediated through these intracellular receptors, which are expressed throughout the central nervous system (CNS) and the periphery and act as transcriptional regulators (Bamberger et al., 1996; Munck et al., 1984). Although the effects of glucocorticoids are generally beneficial, over activation of the HPA-axis may lead to negative symptoms associated with the stress response and eventually develop into pathologies. Examples of negative symptoms of HPA-axis overactivity are insulin resistance that leads to the promotion of visceral adiposity and hypertension, which can develop into diseases of adipose tissue metabolism (Fig.2) (Tsigos and Chrousos, 2002). To prevent this over-activation, glucocorticoids are responsible for inhibiting further HPA-axis activation through GR mediated inhibition of PVN neurons that secrete CRF (McEwen, 1993; Munck et al., 1984). During the absence of a stressor both CRF and AVP are released in the portal system in a circadian rhythm resulting

in differing levels of basal Cort and ACTH at different times of day. CRF and AVP are released in greater amounts in the early morning (in human) stimulating the HPA-axis. The diurnal variations in Cort and ACTH are disrupted by activation of the stress response because the stress response alters their homeostatic balance (Chrousos and Gold, 1998; Horrocks et al., 1990).

An interconnected network of brain nuclei that are simultaneously activated by stress and by CRF activity have been termed the central autonomic system (CAS) (Fig.3) (Saper, 2004). The primary brain structures within the CAS are the central nucleus of the amygdala, bed nucleus of stria terminalis (BST), PVN, the lateral parabrachial nuclei (PBI), locus ceruleus (LC), nucleus of the solitary tract (NTS), and the ventrolateral medulla (VLM). These CRF-rich nuclei are key to the processing of stress-relevant sensory information and generating an adaptive response to a stressor (Justice et al., 2008; Saper, 2004; Swanson et al., 2008).

Catecholaminergic neurons located in the brainstem help to mediate HPA-axis functioning. The NTS conveys incoming sensory information from the cranial nerves to the PVN through long-range projections. Also, the NTS receives projections from key limbic regions that play a role in the behavioral responses to stress including the amygdala and prefrontal cortex (PFC) (Schwaber et al., 1982). Extensive innervation from catecholaminergic neuronal projections to the PVN from stress-responsive neurons in the A2/C2 region of the NTS stimulate the HPA-axis which induces CRF expression and release (Fig.3) (Cunningham et al., 1990; Cunningham and Sawchenko, 1988; Plotsky, 1987; Plotsky et al., 1989; Widmaier et al., 1988). Somatostatin, substance P, and enkephalin are expressed by some neurons located

in the NTS and play a role in the regulation of the HPA-axis as well (Saphier et al., 1994; Sawchenko et al., 1990, 1988). Additionally, nonaminergic NTS neurons send projections to the PVN allowing them to participate in HPA-axis functioning. Also, neurons in the NTS that release glucagon-like peptide 1 are activated by physiological stressors ultimately leading to the release of ACTH via connections to the PVN (Kinzig et al., 2003). Undoubtedly, projections to the PVN from the NTS are critical for the proper functioning and activity of the HPA-axis.

The PFC, hippocampus and amygdala play a major role in glucocorticoid release and behavioral responses to stress (Feldman et al., 1995; Forray and Gysling, 2004; Jacobson and Sapolsky, 1991). These limbic regions do not project to the PVN. It is thought that these regions communicate with the PVN through intermediary neurons located in the BST and brainstem (Herman et al., 2005; Sawchenko et al., 2007). The hippocampus has a role in the termination of the HPA-axis response to stress. Excitation of hippocampal neurons inhibits PVN neuronal activity and decreases glucocorticoid secretion (Rubin et al., 1966; Saphier and Feldman, 1987; Sapolsky et al., 1984). Lesion experiments provided evidence that similar to the hippocampus, the PFC has inhibitory tone on the HPA-axis (Diorio et al., 1993; Figueiredo et al., 2003). However, unlike the hippocampus and prefrontal cortex, the amygdala activates the HPA-axis (Matheson et al., 1971; Van de Kar and Blair, 1999).

Two CRF receptors exist, CRFR1 and CRFR2 (Fig.4) (Dautzenberg and Hauger, 2002). The primary receptor (based on binding affinity and CNS localization) for CRF, CRFR1, is responsible for the anxiogenic effect of CRF. CRFR1 knockout (KO) mice display remarkably reduced anxiety-related behavior and a blunted HPA-

axis leading to the conclusion that CRFR1 is required for the anxiogenic effects of CRF, as well as activation of the HPA-axis (G. W. Smith et al., 1998). Consistent with the receptor's involvement in anxiogenic behavior, CRF KO mice show impaired adrenal responses to various stressors. Additionally, the ACTH response to stress in CRF KO mice is diminished compared to wildtype (WT) mice (Kageyama et al., 2011). However, selective knockout of CRFR1 in various brain systems has led to contrasting effects on anxiety-related behavior. For example, deletion of CRFR1 broadly in excitatory neurons leads to decreased anxiety-related behavior, whereas selective deletion in dopaminergic neurons increases anxiety-related behavior (Refojo et al., 2011).

In contrast, CRFR2 KO mice do not have a clear phenotype compared to the anxiolytic effect of CRFR1 KO mice. CRFR2 deficient mice display normal basal levels of ACTH and Cort providing additional evidence that CRF/CRFR1 signaling is responsible for activation of the HPA-axis. However, CRFR2 KO mice have increased HPA-axis activity in response to a stressor suggesting that CRF/CRFR2 signaling serves to oppose CRFR1-mediated control of ACTH and Cort release (Deussing and Wurst, 2005; Kormos and Gaszner, 2013). Reports have shown that mice lacking the CRFR2 gene have increased anxiety-related behavior compared to WT mice suggesting CRFR2 activation may also serve to reduce anxiety (Kormos and Gaszner, 2013).

CRFR1 is widely expressed throughout the brain including the cortex, hippocampus, brainstem, and external globus pallidus (GPe), while CRFR2 is expressed mainly in the peripheral vasculature and the heart. Additionally, CRFR2 is

expressed in distinct subcortical structures in the brain. CRF binding sites are also found in the adrenal medulla, prostate, liver, kidney, gut, and testes (Bagosi et al., 2006; Justice et al., 2008; Sztainberg et al., 2011; Tsigos and Chrousos, 2002; Van Pett et al., 2000).

CRF is a member of a family of related peptides that include CRF-binding protein (CRF-BP), Urocortin I (UCN1), Urocortin II (UCN2), and Urocortin III (UCN3) (Fig.4) (Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995). UCN1 is expressed in the Edinger-Westphal nucleus (Vaughan et al., 1995). UCN2 expression is localized to the PVN and LC (Reyes et al., 2001). UCN3 expression is more robust being found in the perifornical area of the hypothalamus, BST, lateral septum, and amygdala (Li et al., 2002). CRF has 10-fold higher binding affinity for CRFR1 than CRFR2. In comparison to CRF, UCN1 has 6-fold higher affinity to CRFR1 and a 40-fold higher affinity for CRFR2. UCN2 and UCN3 peptides are selective for CRFR2 (Deussing and Wurst, 2005; Kormos and Gaszner, 2013). This evidence suggests that CRFR2 may be the endogenous receptor for UCN2 and UCN3. In addition, based on binding affinities, it becomes apparent that these related peptides may actually be cooperative agonists and serve related functions. Modulation of this network of peptides is not completely understood.

CRF and UCN1 have related functions through their binding to CRFR1. One study found that UCN1 stimulated ACTH release from the rat anterior pituitary more robustly than CRF by activating CRFR1, both *in vitro* and *in vivo* (Asaba et al., 1998). Secondly, a report looking into hypothalamic GABA release and the effects on the

HPA-axis found that CRF and UCN1 have exactly the same effect (Bagosi et al., 2012).

However, UCN1 has been shown to have distinct functions. In studies using mouse models of Parkinson's Disease (PD), both 6-OHDA (6-hydroxydopamine) and lipopolysaccharide (LPS) mediated nigrostriatal lesion models, UCN1 has been shown to be protective. In fact, when UCN1 was administered at the same time as the toxic agent, or as many as 7 days after the toxic agent, UCN1 is able to prevent and/or rescue dopaminergic neurons once damage has already been established (Abuirmeileh et al., 2007). UCN1 has been shown to mediate cytoprotection in cardiac tissue and in cultured neurons acting through CRFR1 (Brar, 2000; Choi et al., 2006; Scarabelli et al., 2002). Other reports have also pointed to a protective role for UCN1 in the brain and periphery. How UCN1 performs this role through CRFR1 ligand activity is actively being researched. These studies also lead to the question to whether CRF has cytoprotective functions as well. It is likely that the unique actions of UCN1/CRFR1 activity stems from differential activation of secondary messenger networks (discussed below).

UCN2 and UCN3 bind exclusively to CRFR2 therefore different actions than UCN1/CRFR1 signaling would be expected. A study found that intracerebroventricular (ICV) administration of UCN2 produces an increase in amygdalar CRF concentrations after 5 minutes at both low and high concentrations. However, in this paradigm it took 30 minutes for UCN2, and only at high concentrations, to increase hypothalamic CRF concentrations. The same result was found with ICV administration of UCN3. This indicates that UCN2 and UCN3 increase CRF concentrations both in the amygdala

and hypothalamus but in a time-dependent and dose-dependent manner through CRFR2. However, Cort levels were increased in a dose-dependent manner and not a time-dependent manner (Bagosi et al., 2013). In addition to this study, other groups have found dichotomous results on the effects of activation of CRFR2 on the HPA-axis. Several groups have found that activation of CRFR2 stimulates the HPA-axis, but leads to anxiolytic effects in mice (Kishimoto et al. 2000; Bale et al. 2000). In contradiction, other groups have found that ICV administration of UCN2 or UCN3 promotes anxiogenic behavior in rats (Jamieson et al. 2006; Maruyama et al. 2007). To further illustrate the complexity of CRFR2 action, additional studies have reported that ICV administration of UCN2 or UCN3 have no effect on the HPA-axis or behavior (Pelleymounter et al. 2004; Zhao et al. 2007). The difference between these studies is the does of CRFR2 agonist administered. Taking this evidence together, CRFR2 action serves to be either stimulatory or inhibitory on the HPA-axis based on concentration of CRFR2 binding ligand (Bagosi et al., 2013).

CRF-BP binds both CRF and UCN1 with high affinity (Huising and Flik, 2005; Potter et al., 1991; Smith and Vale, 2006; Westphal and Seasholtz, 2006). CRF-BP was identified in maternal plasma where its function was found to inhibit HPA-axis activation as a result of elevated levels of placenta-derived CRF (Linton et al., 1988; McLean and Smith, 2001). CRF-BP dimerizes upon binding to CRF and removes CRF from blood. This CRF removal method serves to protect the maternal pituitary from elevated levels of CRF during pregnancy. In the periphery, CRF-BP is found in the liver and placenta. In the brain, CRF-BP is found in the cortex and subcortical limbic structures (Behan et al., 1995). Coincidentally, CRF-BP is highly expressed in the

pituitary. While the function of CRF-BP is not completely understood, an experiment provided evidence that recombinant CRF-BP reduces CRF-induced ACTH release from anterior pituitary cells *in vitro* (Westphal and Seasholtz, 2006). It is plausible that CRF-BP may bind CRF in the pituitary to lessen CRF receptor activity, reducing the stress response and Cort secretion.

CRFR1 and CRFR2 are class 2B G-protein coupled receptors (GPCRs) which generally couple Gs and activate cAMP production in response to ligand binding. UCN2 and UCN3 minimally induce cAMP production when bound to CRFR2 (Hsu and Hsueh, 2001; Reyes et al., 2001). However, it has also been demonstrated that CRF receptors can activate other secondary messengers including inositol (1,4,5)-tri-phosphate and Ca^{2+} through Gq-coupled activation of phospholipase C (PLC) (Dautzenberg and Hauger, 2002). The complexity of binding affinities and activation of secondary messenger pathways serves to illustrate the multitude of effects the CRF family of related-peptides signaling can have on the CNS and periphery. The focus of this work will be on CRF and CRFR1 signaling in the basal ganglia structure, the GPe which has robust expression of CRFR1.

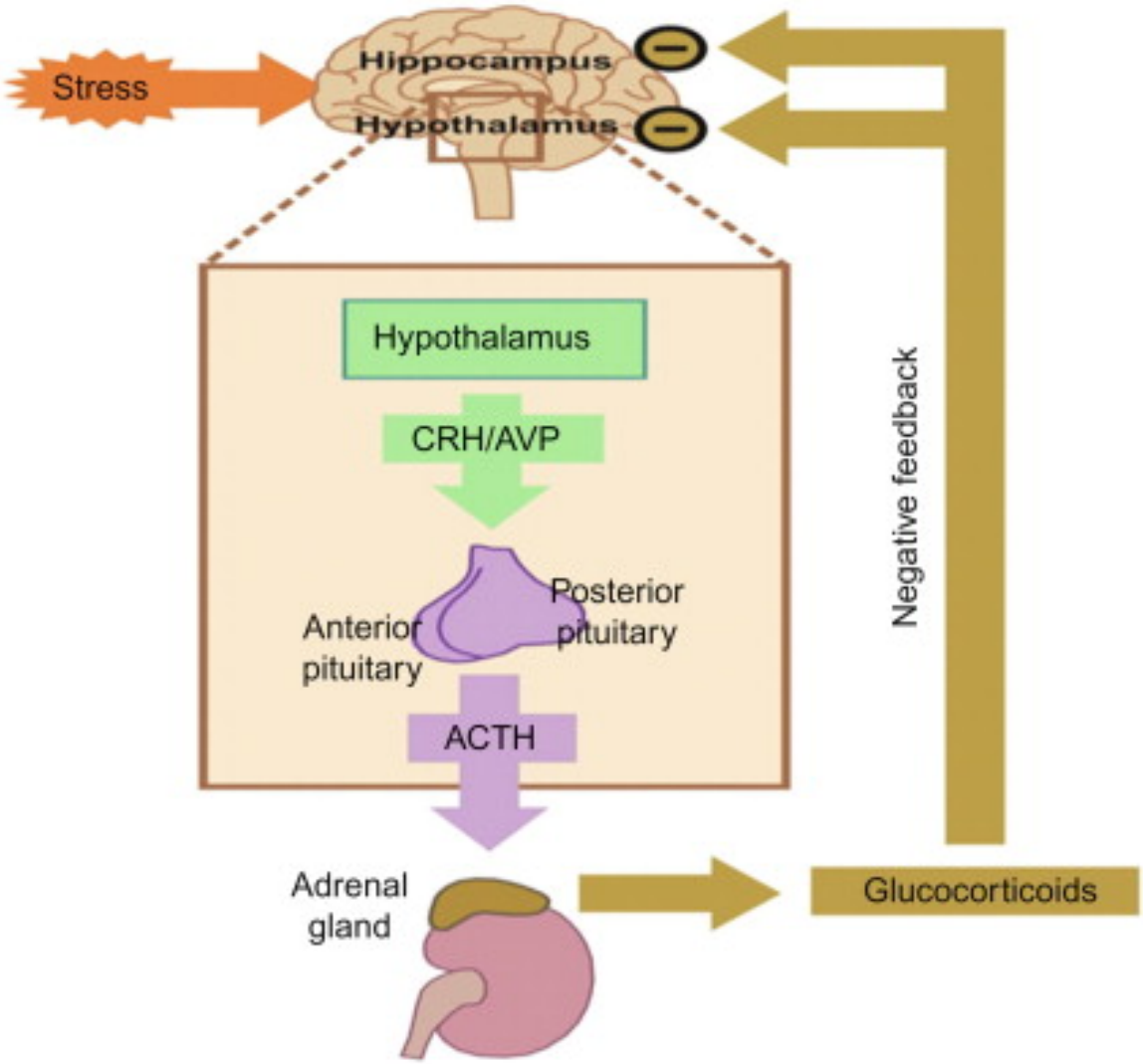
Stress
Hippocampus
Hypothalamus
Hypothalamus
CRH/AVP
Anterior pituitary
Posterior pituitary
ACTH
Adrenal gland
Glucocorticoids
Negative feedback

Figure 1. The Hypothalamic-Pituitary-Adrenal (HPA)-axis. In response to a stressor, CRF is released from the PVN stimulating the release of ACTH from the anterior pituitary. ACTH travels through the systemic circulation to the adrenal cortex and stimulates the release of glucocorticoids, namely cortisol in humans and corticosterone in rodent. Glucocorticoids provide vital negative feedback to regulate the axis. Used with permission from Elsevier. License Number 4047070412252.

Naughton, M., Dinan, T.G., Scott, L.V., 2014. Corticotropin-releasing hormone and the hypothalamic–pituitary–adrenal axis in psychiatric disease, in: Handbook of Clinical Neurology. pp. 69–91. doi:10.1016/b978-0-444-59602-4.00005-8

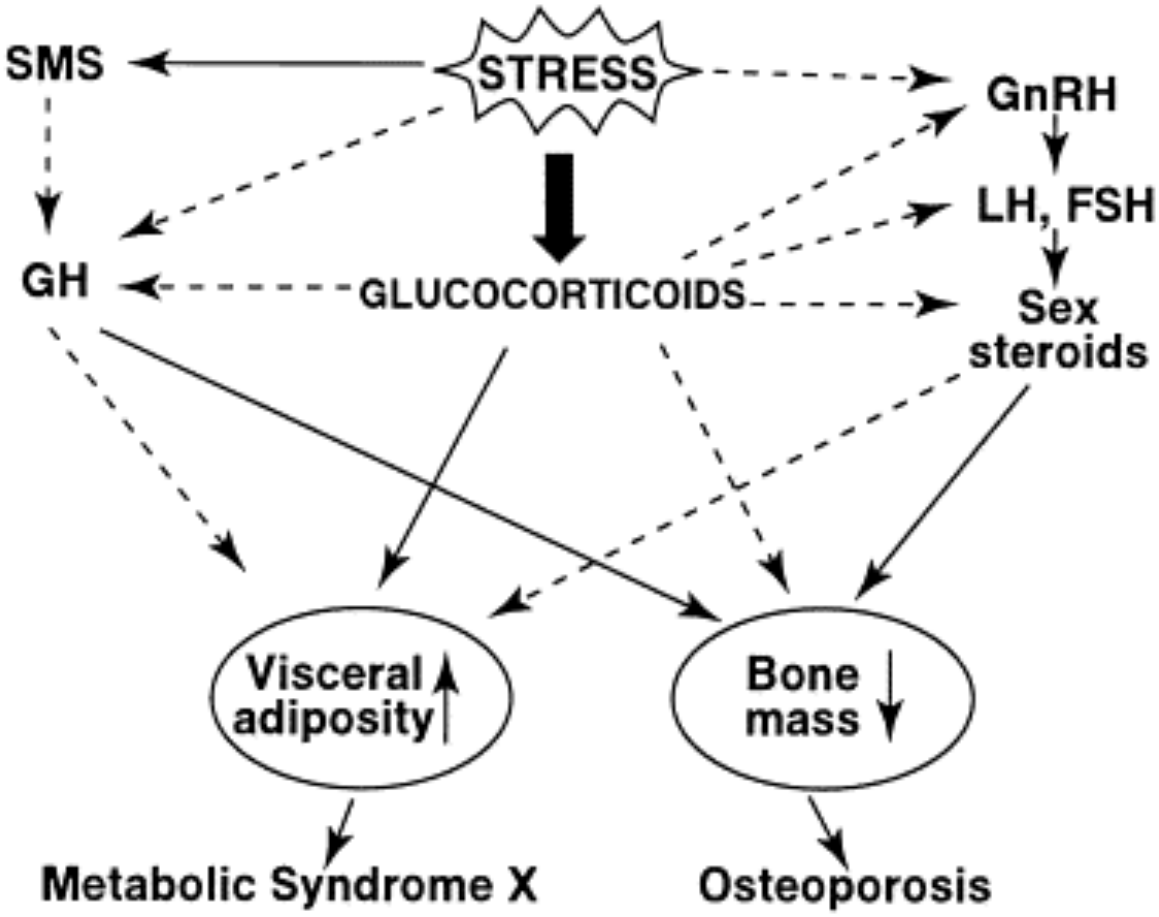
SMS
STRESS
GnRH
LH, FSH
GH
GLUCOCORTICOIDS
Sex steroids
Visceral adiposity
Bone mass
Metabolic Syndrome X
Osteoporosis

Figure 2. Detrimental effects of chronic stress on adipose tissue metabolism and bone mass. Solid lines indicate stimulation; dashed lines indicate inhibition.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871. doi:10.1016/s0022-3999(02)00429-4

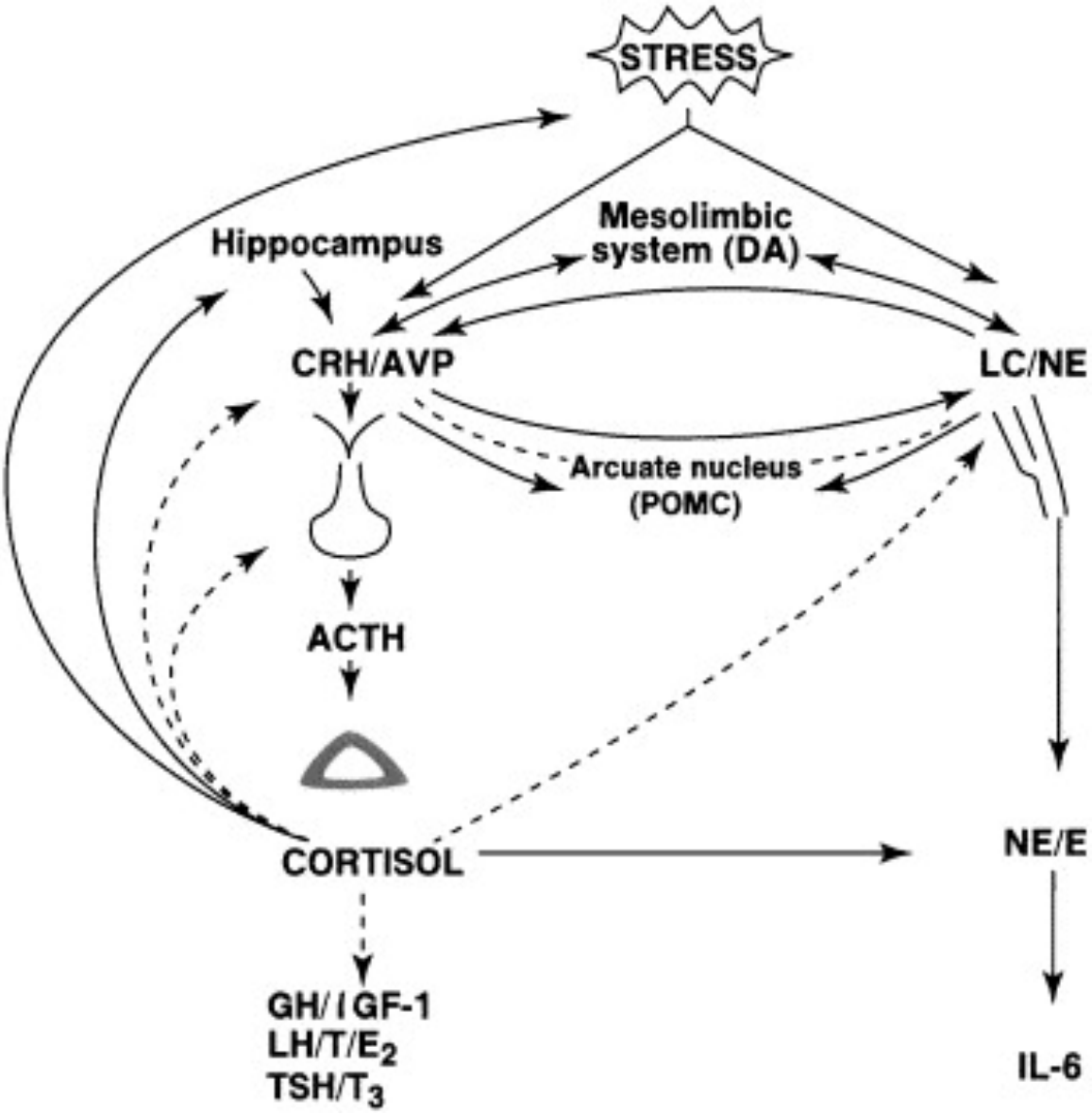
STRESS
Hippocampus
Mesolimbic
system (DA)
CRH/AVP
LC/NE
Arcuate nucleus
(POMC)
ACTH
CORTISOL
NE/E
GH/IGF-1
LH/T/E2
TSH/T3
IL-6

Figure 3. A simplified schematic representation of the central and peripheral components of the stress system, their functional interrelations and their relations to other central systems involved in the stress response. The CRH/ AVP neurons and central catecholaminergic neurons of the LC/NE system reciprocally innervate and activate each other. The HPA axis is controlled by several feedback loops that tend to normalize the time-integrated secretion of cortisol, yet glucocorticoids stimulate the fear centers in the amygdala. Activation of the HPA axis leads to suppression of the GH/IGF- 1, LH/testosterone/E2 and TSH/T3 axes; activation of the sympathetic system increases IL-6 secretion. Solid lines indicate stimulation; dashed lines indicate inhibition.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871. doi:10.1016/s0022-3999(02)00429-4

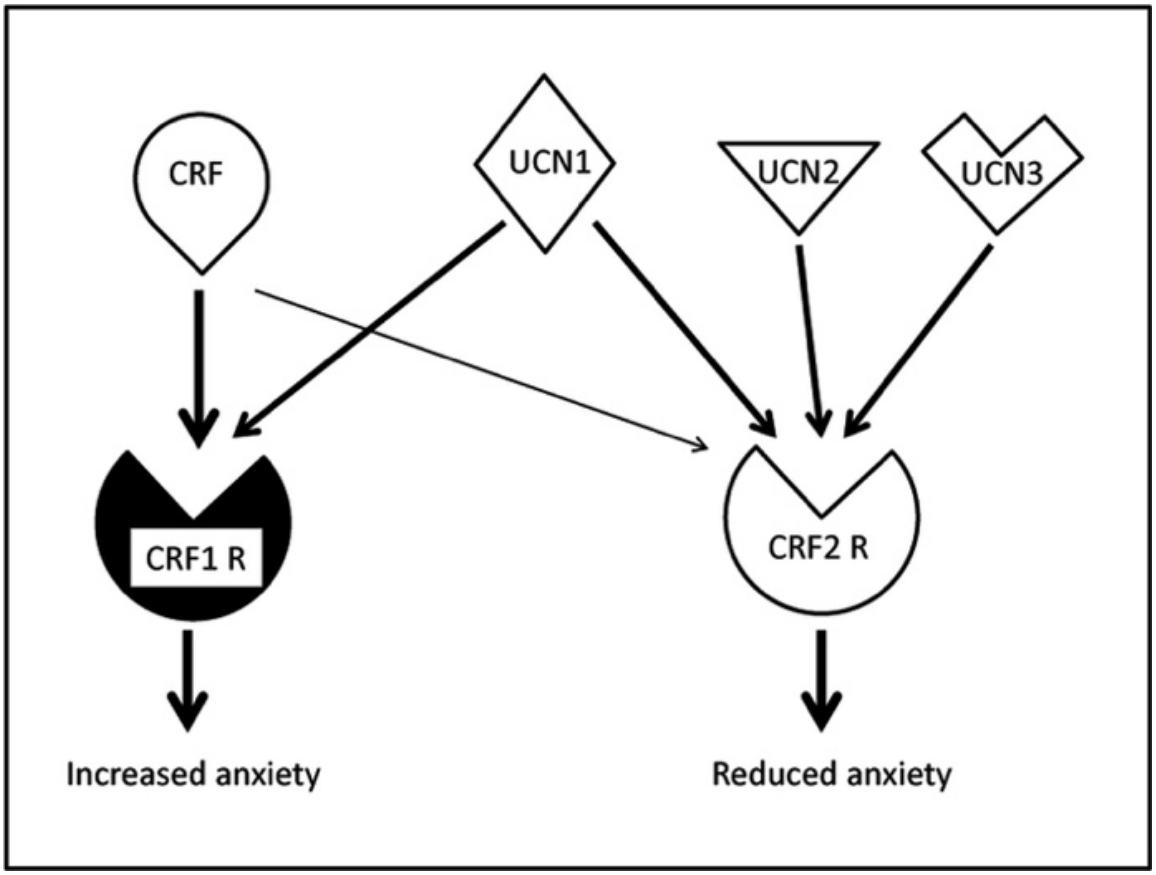
CRF
UCN1
UCN2
UCN3
CRF1 R
CRF2 R
Increased anxiety
Reduced anxiety

Figure 4. The mammalian CRF system and their receptors. CRF binds with high affinity to CRFR1 receptor, however it exerts very low affinity binding to CRFR2. UCN1 has higher affinity for CRFR1 but also binds CRFR2. UCN2 and UCN3 bind with high affinity to the CRFR2 receptor only.

Kormos, V., Gaszner, B., 2013. Role of neuropeptides in anxiety, stress, and depression: from animals to humans. Neuropeptides 47, 401–419. doi:10.1016/j.npep.2013.10.014

The Basal Ganglia and the External Globus Pallidus

The main network of subcortical structures that form the basal ganglia (BG) are the striatum (STR), the globus pallidus (GP), both external GP (GPe) and internal GP (GPi), the subthalamic nucleus (STN), and the substantia nigra: both the pars compacta (SNc) and the pars reticulata (SNr) (Albin et al., 1989; Francois et al., 1984; Yelnik et al., 1987). The STR is composed of projection neurons. Studies in rats have suggested that striatal projections form dense collateral projections with axons from a single neuron terminating in the GPe, GPi, and SNr (Graybiel et al., 1979; Grofová, 1979). The STR receives input from all of the cortex (Kemp and Powell, 1970; Young et al., 1981). The primary neurotransmitter of the STR, GPe, GPi, and SNr is GABA, while the primary neurotransmitter of the STN is glutamate. The primary neurotransmitter released from the SNc is dopamine (Nakanishi et al., 1987; Smith and Parent, 1988). The GPi and SNr are the major output nuclei of the BG.

The classical connectivity of the basal ganglia is divided into a direct, motor promoting, and an indirect, motor inhibiting pathway which originate from distinct populations of striatal medium spiny neurons (MSNs) and project to different output structures, GPi or SNr (Fig.5) (Calabresi et al., 2014). However, current knowledge about the basal ganglia can be explained in an oversimplified, but useful, direct/indirect pathway model or in much greater detail that many have deemed too complex and controversial (Calabresi et al., 2014).

There are as many hypotheses about the functions of the basal ganglia as groups who study its connectivity. In particular, the study of PD has had a major influence on the speculation of basal ganglia function. As parkinsonian patients have

difficulty initiating movement, this led to the hypothesis that the BG is involved in the automatic execution of learned motor movements (Albin et al., 1989; Turner and Desmurget, 2010). Overall, early studies into the functions of the BG were based on the dysfunction caused by disorders of the BG which are primarily motor oriented; PD, dystonia, and Huntington's disease. Early studies using ablation of the output nuclei of the BG provided better patient outcomes in PD than ablation of BG inputs leading many to believe that dysfunction in BG circuits is more disruptive than eliminating the output of the BG in totality (Albin et al., 1989; Turner and Desmurget, 2010). This indicates that dysfunctional input to the STR may be the primary pathology that leads to disorders of the BG.

The balance between the direct and indirect pathway is regulated by the opposing actions of dopamine on excitatory D1 receptors and inhibitory D2 receptors in the STR which receives differential dopaminergic innervation from the SNc. Importantly, these pathways do not function individually; both pathways are active simultaneously. Dopamine increases the activity of the direct pathway by D1 receptor excitatory activation while dopamine action on D2 inhibitory receptors reduces activity in the indirect pathway. The result of this particular innervation pattern is a reduction in the signaling from the output nuclei of the BG, the GPi and the SNr, manifesting as increased movement. Due to this circuitry, an increase in dopamine release from the SNc causes increased activity in the direct pathway resulting in increased movement while a decrease in dopamine release causes decreased activity in the direct pathway and increased activity in the indirect pathway resulting in decreased movement (DeLong and Wichmann, 2007). This model serves particularly well to describe the

manifestations of PD. As dopaminergic neuron loss occurs in the SNc, striatal dopamine depletion occurs leading to the motor symptoms of PD. Also, this model explains the efficacy of the surgical interventions and drugs used to reduce PD symptoms such as lesion of the GPi. By reducing the functioning of the GPi, less GABA is released to the thalamus allowing it to excite the motor cortex and increase movement. Additionally, treatment with L-dopa to replace lost dopamine increases the activity of the motor promoting, direct pathway.

In the 1970s, it was thought that the BG integrated projections from limited portions of the cortex and projected this input through the thalamus to the motor cortex and supplementary motor area. This knowledge provided a mechanism by which movement could originate from functional cortical areas (DeLong and Wichmann, 2007). More recent studies have suggested that the BG receives input from segregated cortical areas. This input terminates in specific basal ganglia territories which is then transmitted to the thalamus in a region specific pattern. Finally, the regional specificity of the thalamic connections project back to the same regions of the cortex from which the input originated (Alexander et al., 1986; DeLong and Georgopoulos, 2011; Kelly and Strick, 2004). These theories are partially based on experiments using rabies virus that revealed multisynaptic circuits generating segregated loops and lead to current models of the basal ganglia as segregated, specific cortico-subcortical loops (Kelly and Strick, 2004). For example, each of the BG nuclei have topologically organized motor, associative, and limbic circuits as revealed by imaging studies in humans (Fig.6) (Draganski et al., 2008; Lambert et al., 2012; Lehéricy et al., 2004). If I take these new theories into account, disorders of the

BG are actually pathologies generated by aberrant neuronal activity throughout a specific loop (DeLong and Wichmann, 2007). As the complexities and functions of the BG are vast and debatable, I will focus on a specific nucleus of the BG located in the motor inhibiting, indirect pathway; the GPe. As mentioned, the GPe expresses high levels of the CRF receptor CRFR1 making it of particular interest in my examination of the stress response and CRF/CRFR1 signaling.

For many years, the GPe was considered a homogenous GABAergic nucleus with the function of relaying inhibitory input from the STR downstream to the glutamatergic STN. In turn, the STN increases GPi activity leading to decreased movement (Albin et al., 1989; Alexander and Crutcher, 1990; Calabresi et al., 2014; Y. Smith et al., 1998; Wichmann and DeLong, 1996). However, in a historic experiment in awake macaque, DeLong found that the nucleus was not homogenous. He found that there are two subtypes of GABAergic neurons based on electrophysiological properties (DeLong, 1971). He found “high-frequency pausers” (~80%) which are neurons that fire at high frequency for long periods of time (~30 min) followed by brief periods of silence or low frequency firing. Also, he found “low-frequency bursters” (~20%), which fire at low frequency for long periods of time followed by quick bursts of high frequency firing. This was the first evidence that there are two populations of GABAergic neurons in the GPe with distinct properties (DeLong, 1971). Today, the electrophysiologically distinct GPe neuronal subtypes are referred to as prototypic neurons closely resembling the “high-frequency pausers” and arkypallidal neurons, which resemble the “low-frequency bursters” (Abdi et al., 2015; Hernández et al., 2015; Mallet et al., 2012, 2008).

Prototypic neurons and arkypallidal neurons can be distinguished by their active and intrinsic membrane properties and *in vivo* responses to movement (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015; Saunders et al., 2016). Also, prototypic and arkypallidal neurons are derived from distinct neuronal lineages, express distinct molecular markers, and have distinct projection sites. Prototypic neurons are derived from the medial ganglionic eminence and express the transcription factor Nkx2.1 and the calcium binding protein parvalbumin (PV) (Flandin et al., 2010; Mallet et al., 2012). Arkypallidal neurons are derived from the lateral ganglionic eminence and express FoxP2, preproenkephalin (PPE), and Npas1 (Dodson et al., 2015; Mallet et al., 2012; Glajch et al., 2016). Interestingly, co-expression of Npas1 and FoxP2 confers arkypallidal neuronal phenotype, while expression of Npas1 alone confers a prototypic neuronal phenotype (Abdi et al., 2015; Hernández et al., 2015). Finally, while Nkx2.1/PV prototypic neurons target the STN, arkypallidal neurons only innervate the STR (Fujiyama et al., 2016; Mallet et al., 2012).

Motor deficits as manifested in PD that result from GPe dysfunction confirm its role in sensorimotor function (Anderson and Horak, 1985; Dodson et al., 2015; Jaeger et al., 1995; Jin et al., 2014; Kita, 2007; Mallet et al., 2016; Mitchell et al., 1987; Nambu et al., 2002; Obeso et al., 2006; Tachibana et al., 2011; Turner and Anderson, 2005; Yoshida and Tanaka, 2016). With a confirmed role in sensorimotor function, it is now believed that the GPe is a central nucleus in the BG (Fig.5) (Mallet et al., 2012; Mink, 1996; Parent and Hazrati, 1995). Multiple experiments have been performed in 6-OHDA lesioned PD rodent models to examine how aberrant GPe functioning effects

PD symptomology. Evidence has been provided by cell-labeled electrophysiology that PV prototypic neuron firing is preserved in PD models while Npas1 neuron firing is decreased (Abdi et al., 2015; Hernández et al., 2015). In fact, Npas1 neurons which primarily fall into the arkypallidal subtype, synapse on spiny projection neurons (SPNs) and interneurons (INTs) in the STR where they strengthen their connections in 6-OHDA lesioned PD models (Glajch et al., 2016). This evidence provided the first insight that the GPe projecting upstream to the STR contributes to motor dysfunction in PD. From these evidence, the GPe has a central role in BG circuitry and is greatly altered in the dopamine depleted brain as a result of the progression of PD. Intriguingly, the GPe has robust expression of the CRF receptor CRFR1 suggesting that it may play a role in the stress response. In this work, I will explore the connectivity of the stress system with the GPe and the functional outcomes on behavior as a result of activation of CRFR1 expressing neurons in the GPe.

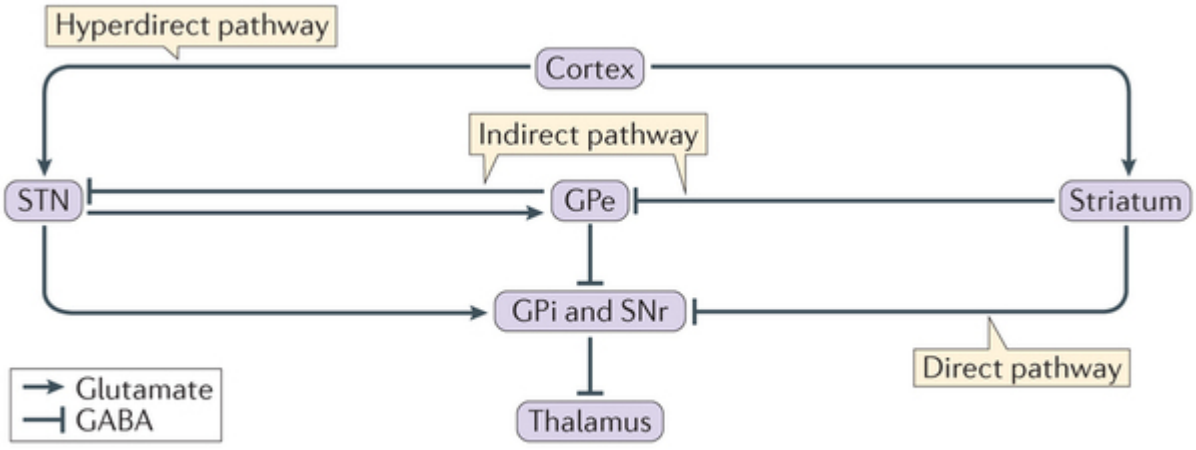

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Figure 5. The hyperdirect, direct and indirect pathways and neural inhibition in the cortico–basal ganglia circuits.

Jahanshahi, M., Obeso, I., Rothwell, J.C., Obeso, J.A., 2015. A fronto–striato–subthalamic–pallidal network for goal-directed and habitual inhibition. Nat. Rev. Neurosci. 16, 719–732. doi:10.1038/nrn4038

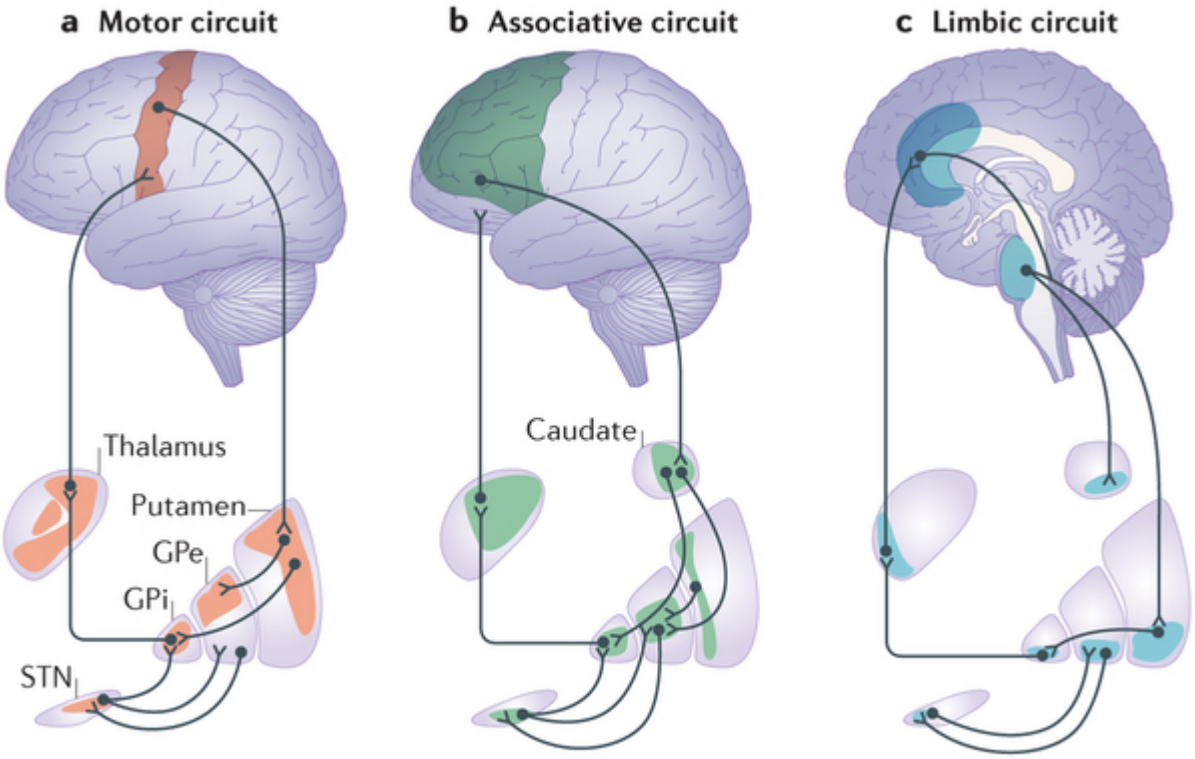
a Motor circuit
b Associative circuit
c Limbic circuit
Thalamus
Putamen
GPe
GPi
STN
Caudate
Nature Reviews | Neuroscience

Figure 6. The main functional subdivisions in the sensorimotor, associative and limbic cortico–striatal circuits. These multiple loops suggest that the GPe map play a role in all of these complex behaviors (A) Motor circuit (B) Associative Circuit. (C) Limbic circuit.

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Jahanshahi, M., Obeso, I., Rothwell, J.C., Obeso, J.A., 2015. A fronto–striato–subthalamic–pallidal network for goal-directed and habitual inhibition. Nat. Rev. Neurosci. 16, 719–732. doi:10.1038/nrn4038

Computational and Theoretical Neuroscience aspects of the External Globus Pallidus

The BG has a myriad of proposed (theoretical) functions in behavior and cognition. One function is action selection and the GPe is believed to play a major role in this behavior (Berns and Sejnowski, 1998; Bogacz et al., 2016; Cools, 1980; Denny-Brown and Yanagisawa, 1976; Hikosaka, 1994; Mink, 1996; Redgrave et al., 1999; Robbins and Brown, 1990; Schultz, 2001; Wickens, 1997, 1993). A "selection problem" occurs when two or more competing behavior programs seek access to a restricted or limited network such as the BG (Redgrave et al., 1999). The selection of one behavior over another is sometimes referred to as behavior switching (McFarland, 1989; Redgrave et al., 1999). When multiple actions can be taken, the one with the greatest salience should be selected. For instance, if an animal has a choice between eating, drinking, or escaping a threat, escaping the threat should be able to cancel the other actions as it is the most salient for survival. All three behaviors require the motor system which is a limited resource. A mechanism to inhibit the less salient behaviors must exist. In this case, the mechanism is the ability of the BG to inhibit actions or behaviors.

Computational models have provided evidence as to how the BG could select an action in the fastest way possible with the greatest accuracy. These models further suggests that a single nuclei in the BG, the GPe, must calculate a function of its inputs (Bogacz et al., 2016).

Goal-directed behavior is a type of action or behavior an animal can select. Goal-directed behaviors are intentional, considered, and sensitive to the value of the prospective goal (Dickinson, 1985). In contrast, the opposite of goal-directed behavior

is a response automatically triggered by some stimuli. This is referred to as habitual behavior (Gillan and Robbins, 2014). Animals use both reflective (goal-directed) and reflexive (habitual) action selection (Dolan and Dayan, 2013; Seger and Spiering, 2011). For goal-directed behaviors, it is critical that an animal is able to select the appropriate action and learn to modify it irrespective of whether it is motor or non-motor in nature (Yttri and Dudman, 2016). Interestingly, goal-directed behavior suffers in times of stress (Schwabe and Wolf, 2009). It is possible that this decrease in goal-directed behavior may be mediated directly by CRF actions on the GPe as the stress response may be the most salient action to select, allowing behavioral switching from goal-directed to habitual or automatic behavior.

In a recent computational model, the STN-GPe network regulates the competition between actions and guarantees that the most beneficial action is selected. The GPe needs to provide GABAergic feedback to the STN in order to compute the optimal action. It is postulated that prototypic and arkypallidal neurons that make up the GPe network could compute the function required for optimal action selection. The model calculates that the GPe will receive input from the STN even when the STN does not receive excitatory input.

Recent modeling of the connectivity in the STN-GPe network suggests that both prototypic and arkypallidal neurons receive input from the STN. The model considers both GPe neuronal subtypes, prototypic and arkypallidal neurons. The micro circuitry of the GPe processes the input from the STN allowing for optimal action selection. The STR provides input to both prototypic and arkypallidal GPe neurons. However, the model postulates that prototypic neurons receive more input from the

STR than arkypallidal neurons. Ultimately, the model proposes that the GPe itself, based on the different electrophysiological properties of prototypic and arkypallidal neurons and their projections sites, is able to calculate optimal action selection (Bogacz et al., 2016).

Although computational modeling is a very beneficial tool to make predictions in the study of theoretical and computational neuroscience, the caveats that it may or may not be reproducible *in vivo* exists. However, this evidence bolsters my investigation into CRFR1 activity in the GPe because the possibility of optimal action selection in the stress response when I explore activation of CRFR1 expressing neurons exists. The computational modeling provides an intellectual framework for conceptualizing how CRF stress signaling could mediate behavior switching through its novel actions on the GPe.

Opening Remarks

Interestingly, CRFR1 is highly expressed in the GPe but no function has been defined (Justice et al., 2008). CRF is the stress peptide that initiates the stress response. The question is raised as to why the receptor responsible for the anxiogenic effects of CRF is located in the GPe. One study, found that knockdown of CRFR1 by siRNA injection directly into the GPe leads to an increase in anxiety-related behavior therefore concluding that the GPe may have an anxiolytic role in behavior (Sztainberg et al., 2011). This is in direct opposition of the known function of CRF/CRFR1 signaling. As has been discussed throughout this introduction, BG functions are not clear and many have been proposed. Through this work, I will provide evidence that the GPe has a role in anxiety-related behavior. Assigning a new role or circuit of the BG to the expression of complex behaviors advances our knowledge of the BG as more than merely a motor-mediating network. My novel findings demonstrate that one subtype of neuron in the GPe that expresses CRFR1 modulates anxiety-related behavior as part of an integrated stress response. Also, I find that neurons that express CRFR1 are exclusively prototypic. As the GPe has robust expression of CRFR1, experiments were performed to understand how CRF circuits may impinge on BG functioning to bias motor output or action selection in the stress response.

In this dissertation, I document the description of CRFR1 expressing neuron's electrophysiological response *ex vivo* in acute brain slices to CRF application. I also systematically characterize the molecular phenotype of CRFR1 expressing neurons. I demonstrate the connectivity of CRF neurons to CRFR1 expressing neurons in the GPe and evaluate the local connectivity within the GPe. Lastly, I explore the functional

role of CRFR1 neuron activation, using a chemogenetic strategy, in the GPe on anxiety-related behavior while assaying mouse behavior in a variety of anxiety-related behavior and movement paradigms.

Experimental Procedures

Animal care and genetic crosses

Mice were housed up to five per cage with *ad libitum* access to food and water in a pathogen-free facility with a 12h light/dark cycle. All procedures were performed in accordance with the National Institutes of Health guidelines and with the approval of the University of Texas Health Science Center at Houston Institutional Animal Care and Use Committee (IACUC). Male and female mice aged 12-16 weeks were used for viral tracing and immunohistochemistry experiments. *Ex vivo* electrophysiological recordings were performed in 4-8 week old animals. Behavioral experiments were performed using 12-16 week old male mice. The BAC transgenic mouse line, CRFR1-GFP, was used to accurately identify CRFR1 expressing neurons for immunohistochemistry and electrophysiological recordings. In brief, an approximately 500-bp fragment of genomic DNA directly upstream of the ATG start codon in CRFR1 was amplified by PCR and sub-cloned in a shuttle vector containing eGFP with a polyA encoding sequence. A 500-bp DNA sequence within the first intron of CRFR1 was sub-cloned into the shuttle vector. The modified shuttle vector was transformed into *Escherichia coli* containing the BAC rp23-4B21. This BAC contained the CRFR1 genomic locus. BAC DNA from a single *E.* coli colony was isolated, and PCR was performed to verify that the shuttle vector inserted into the CRFR1 locus by homologous recombination. DNA that showed correct recombination was isolated and injected into single-cell mouse oocytes. A single mouse carrying the BAC was obtained (Justice et al., 2008). A CRFR1-Cre-2A-^{td}tomato BAC transgenic mouse line was generated using a similar strategy to the CRFR1-GFP BAC transgenic mouse line. Briefly, a BAC (rp23-239f10; Children's Hospital Oakland Research Institute)

containing the entire genomic locus of CRFR1 was modified using recombineering (Chan et al., 2007; Liu et al., 2003). A cassette encoding iCre-p2A-^{td}tomato-PA was inserted in the first exon at the site of the ATG start codon for CRFR1. Modified BAC DNA was purified and injected in single celled oocytes to generate transgenic offspring (Genetically Engineered Mouse Core, Baylor College of Medicine, Houston). Three independent transgenic lines were recovered, of which one (CRFR1-cre) had the best co-localization of cre/^{td}tomato with CRFR1-GFP. *In situ* hybridization was performed and confirmed that *iCre* is expressed in the same cells as *Crfr1* in the Globus Pallidus. CRFR1-cre mice were used for viral tracing and behavioral experiments. The BAC transgenic Npas1-iCre-2A-^{td}tomato mouse line herein referred to as Npas1-cre has been previously characterized and validated (Hernández et al., 2015). CRH-IRES-CRE (CRF-cre; (Chen et al., 2015)) was obtained from The Jackson Laboratory and crossed to a floxed ^{td}tomato reporter line (Ai9; (Madisen et al., 2010)) to express the fluorescent reporter ^{td}tomato in CRF neurons and fibers.

Tissue preparation, immunohistochemistry, and cell quantification

Mice were deeply anesthetized by IP injection of tribromoethanol (125 mg/kg from a stock solution of 1.25% v/v) and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in the same fixative for 12 h at 4℃. Brains were then equilibrated in 30% sucrose, and 30 μm coronal sections were prepared using a frozen sliding microtome. Free floating sections were washed in PBS and incubated overnight with antibodies diluted in PBS + 0.4% Triton X-100 with 2% normal donkey serum. Antibodies used were as follows: goat anti-GFP (1:1000, Rockland), rabbit anti-TTF-1 (also known as Nkx2.1; 1:1000, EMD Millipore),

rabbit anti-NeuN (D3S3I,1:1000, Cell Signaling Technologies), mouse anti-Parvalbumin (1:1000, EMD Millipore), rabbit anti-Npas1 (Npas1-3, 1:1000, gift from S. Chan), rabbit anti-FoxP2 (1:1000, EMD Millipore), rabbit anti-Synapsin-1 (D12G5, 1:1000, Cell Signaling Technologies), rabbit anti-c-FOS (1:20,000, Synaptic Systems), and rabbit anti-CRF (rc70, 1:2000, gift from W. Vale). Following incubation, tissue sections were washed in PBS and treated with the appropriate secondary for 2 h, washed in PBS, and mounted on gelatin-coated slides. Images were obtained on a Leica TCS SP5 confocal microscope. All images presented are max projections. In CRFR1-GFP mice, colocalization of GFP and the molecular marker of interest was quantified by manual counting of 3 images per brain from n=3-5 mice at different rostro-caudal levels of the GPe. Quantification was averaged over all sections without separation based on rostro-caudal distribution of neurons expressing molecular markers as previously described (Dodson et al., 2015). For tracing experiments, half of the brain sections were mounted for quantification of starter and traced cells using an epifluorescent microscope. Staining of starter and traced cells was performed on brain sections not used for connectivity ratio quantification.

Stereotaxic injections of adeno-associated virus

Mice were deeply anesthetized with isoflurane and secured in a mouse stereotaxic frame. Under aseptic conditions, a minimal incision was made to expose the skull. A small craniotomy was made using a drill at coordinates 0.0 mm rostral, ± 2.1 mm lateral, and -3.8 mm ventral to bregma. Injections were made using a Nanoject II (Drummond Scientific) with a pulled glass pipette at a rate of 50 nL/min. Following completion of injection, the pipette was left in place for 5 minutes to allow for

absorption of the virus before being removed. For viral tracing, I injected 50 nL of a 1:1 mixture of Cre-dependent AAVs carrying FLEXed TVA-mCherry and FLEXed Rabies G (AAV-EF1a-FLEX-TVA-mCherry, AAV-CA-FLEX-G obtained from the UNC viral vector core; (Watabe-Uchida et al., 2012). After 2 weeks, I delivered 200 nL of Pseudotyped rabies virus (AAV-EnvA-SADΔG-eGFP supplied by Dr. Ben Arenkiel; (Wickersham et al., 2007)) in the same location. After 5 days, I made an icv injection of colchicine (0.3 mg/kg solution in ddH_2O) to enhance CRF staining of cell bodies. Mice were sacrificed at 7 days post-injection of PTRV. For DREADD experiments, I made 100 nL bilateral injections of Cre-dependent Gq-coupled DREADD (AAV-hSyn-DIO-hM3D(Gq)-mCherry obtained from the UNC Viral Vector Core; (Armbruster et al., 2007)) into the GPe (0.0AP, ± 2.1ML, -3.8DV to bregma). Mice were returned to their home cage for 2 weeks before initiation of behavioral testing to allow for expression of DREADDs from the virus.

Slice preparation

Parasagittal slices (250 μm) were obtained from male and female CRFR1-GFP animals, aged 4-8 weeks. All procedures followed were in accordance with National Institutes of Health guidelines and approved by the University of Texas Health Science Center at Houston animal welfare committee. Animals were anesthetized using isoflurane, decapitated, and their brains were quickly extracted and placed into ice-cold solution saturated with 95% O_2–5% CO_2, and consisting of the following (in mM): 212 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 26 $NaHCO_3$, 11 glucose, and 0.5 $CaCl_2$. Slices were prepared using a vibratome (Leica VT1200S) and then incubated at 34°C for 50 min in artificial cerebrospinal fluid (ACSF) containing the following (in

mM): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$ (saturated with 95% O_2–5% CO_2). Slices were then kept at room temperature to preserve health until used for experiments.

Electrophysiological recordings

Recordings were performed at 31–33°C using an in-line heater (Warner Instruments) while perfusing the recording chamber with ACSF. Neurons were visualized using infrared differential interference contrast using an Olympus BX51WI microscope and an IR-1000 camera (Dage-MTI). GFP+ cells were identified under fluorescence using an X-Cite 120Q light source. Loose-patch voltage-clamp recordings from CRFR1-GFP+ and GFP- GPe neurons were obtained with glass pipettes (3–5 MΩ) filled with ACSF. Seal resistance was maintained between 40–70 MΩ. To minimize perturbations of the firing rate of the recorded neuron, command voltage was continuously adjusted during the recordings to maintain a holding current near 0 pA (Perkins, 2006). Experiments were done in the presence of DNQX (10 μM), D-APV (25 μM), and Picrotoxin (50 μM) to block AMPARs, NMDARs, and GABAARs, respectively. DNQX and Antalarmin were obtained from Tocris Bioscience. Picrotoxin and D-APV were obtained from R&D Systems. CRF was kindly provided by J. Rivier. All other chemicals were obtained from Sigma-Aldrich.

Data acquisition and analysis

Data were acquired using a Multiclamp 700B amplifier (Molecular Devices), filtered at 6–10 kHz, and digitized at 20 kHz with a 16-bit analog-to-digital converter (Digidata 1440A; Molecular Devices). Recordings were acquired using Clampex 10.3

software (Molecular Devices) and analyzed using custom macros written in Igor Pro (Wavemetrics).

Calculation of Pseudotyped Rabies Virus "Connectivity Ratio"

Brain sections were imaged and "starter" neurons were identified by their co-expression of TVA-mCherry and GFP indicating a cell competent to transmit the rabies virus. Traced neurons were identified by their expression of GFP in the absence of TVA-mCherry. A calculation was made of total traced neurons in a given region divided by the total starter neurons in the GPe to give the "connectivity ratio" used to compare the relative cellular input from canonical and novel brain regions that project and synapse in the GPe. This "connectivity ratio" allowed for the comparison of GPe inputs between CRFR1-cre and Npas1-cre animals.

Behavioral Testing

Prior to behavioral testing, mice were placed in the behavior room and allowed to acclimate for 1 h. At the beginning of testing, 2 mg/kg of Clozapine-N-oxide (CNO) was IP injected, and the mice were returned to their home cage for a period of 10 min to allow for the activation of DREADDs. Mice were tested on the Light-Dark Transfer (LDT) test, Marble Burying task, and the Open Field Assay (OFA). All parameters of movement were tracked by a computer system and analyzed *post-hoc*.

OFA. Mice were placed in the center of 36 cm^2 square open field and allowed to move freely for 30 min. Their field position was tracked using a photo beam system (Kinder Scientific). The maze was divided into a surround zone (9 cm. bordering the edge of the field on all sides), and a center zone (center 18 cm. square field). I analyzed total distance traveled, center distance traveled, surround distance traveled,

center time, and surround time. I used the ratio of center distance to total distance traveled as a measurement of anxiety-related behavior to normalize for movement differences within each mouse, to minimize the impact of changes in total movement on anxiety scores (Bale et al., 2000).

LDT. Mice were placed in the OFA with a Plexiglass insert dividing the arena into a dark half and a light half. The light and dark regions were separated by a cut out allowing the mouse to pass freely from one side of the arena to the other. Mice were placed on the dark side at the beginning of the 10 min testing period. I measured total distance traveled, light distance, dark distance, number of transfers between regions, and time spent in each region. I quantified anxiety-related behavior using light entries and total time spent in the light (Bale et al., 2000).

Marble Burying. Mice were placed in a Plexiglass cage of the same dimensions as their home cage filled with 1 liter of alpha-dri bedding. 10 marbles were placed in the cage spaced evenly apart on the bedding. The testing period lasted for 15 minutes. At the completion of testing, only marbles that were completely buried were counted as buried. An increase in the total number of marbles buried is interpreted as an increase in anxiety-related behavior (Kedia and Chattarji, 2014).

Post-hoc analysis of viral injections

Immediately following the completion of behavior analysis, mice were perfused, brains sectioned and mounted for analysis using an epifluorescence microscope to determine the accuracy and relative diffusion of viral injections. Mice were excluded from the study (~10) if neurons outside of the target region (GPe) expressed DREADD-mCherry. In some cases, animals were given injections of CNO (2 mg/kg

IP) 2 hours before perfusion and brain slices were stained for the immediate early gene cFOS to verify CNO activation of DREADD expressing neurons. No mice were excluded from this analysis.

Statistics

For behavioral experiments (control vs. DREADD, same genotype), a two-tailed Student's *t*-test was used to determine significance in GraphPad Prism 5.04. For slice electrophysiology, a two-tailed Student's *t*-test was used. The results are presented as mean ± SEM. For all figures $p<0.05$ - *, $p<0.01$ - **, and $p<0.001$ - ***.

CRFR1-GFP Neuronal Electrophysiological Profile in Response to CRF

In Brief

There have been no functional studies performed to validate GFP as a proxy for CRFR1 expression in the GPe in the BAC transgenic CRFR1-GFP mouse. CRFR1 activation by the stress-responsive neuropeptide CRF is accepted to be excitatory, however sex difference studies in CRF/CRFR1 signaling suggest this doctrine may be weakening. One study found that serotonergic neurons in female mice have reduced excitability and a decreased response to CRF compared to males suggesting that CRF may not always be excitatory when considering both males and females. In addition, it was found that CRF infusion into the dorsal raphe (DR) in male mice increases cFos, an immediate early gene whose expression is indicative of neuronal activation. While CRF activates neurons in the DR of males, infusion of CRF into the DR of female mice decreases cFos, indicating the absence of neuronal activation (Bagosi et al., 2012; Howerton et al., 2014).

This is the first study to record the electrophysiological response of CRFR1+ GPe neurons to CRF application. In order to provide evidence that GFP can report the expression of CRFR1 and that CRF is excitatory through CRFR1 in the GPe, we performed electrophysiological recordings from GFP+ neurons in the GPe of CRFR1-GFP mice while infusing a single concentration of CRF into the recording chamber. We performed simultaneous paired recordings. Using paired recordings provided an unparalleled control as both GFP+ and GFP- neurons are being recorded simultaneously as CRF was applied to the same brain slice. Our results indicate that 100% of the CRFR1-GFP+ neurons respond to bath application of CRF with a robust increase in firing. 100% of the paired neurons that did not express GFP did not

respond to bath application of CRF. The excitatory effect of CRF on GFP+ neurons was blocked using the small molecular, non-peptide CRFR1-specific antagonist Antalarmin, verifying that the effects of CRF were in fact mediated through CRFR1.

Results

To investigate the impact of CRF on GPe neuron excitability, we prepared parasagittal brain slices from CRFR1-GFP animals and performed simultaneous loose-patch recordings from neighboring GFP+ and GFP- neurons in the GPe. We blocked fast glutamatergic and GABAergic synaptic transmission with 10 μM DNQX, 25 μM AP5, and 50 μM Picrotoxin to examine the effects of CRF on autonomous activity of GPe neurons. Following bath application of 30 nM CRF, GFP+ neurons displayed a significant increase in mean firing rate (47.56 ± 6.07 Hz vs. 25.84 ± 8.86 Hz, n=5, $t_{(4)}$=2.88, $p<0.05$, Fig.7 A top trace) while simultaneously recorded GFP- neurons were unaffected by the application of CRF (12.01 ± 3.85 Hz vs. 12.35 ± 3.88 Hz, n=5, $t_{(4)}$=0.34, p=0.75, Fig.7 A bottom trace). To ensure that the observed response to CRF in GFP+ cells was mediated by CRFR1, we recorded from GFP+ cells in the presence of the selective CRFR1 non-peptide antagonist Antalarmin (Webster, 1996). In the presence of 1 μM Antalarmin, CRF application did not increase the mean firing rate of GFP+ neurons (21.99 ± 7.61 Hz vs. 19.02 ± 6.06 Hz, n=5, $t_{(4)}$=0.92, p=0.41, Fig.7 B). These data confirm that GFP expression in CRFR1-GFP mice reports functional expression of CRFR1 in the GPe.

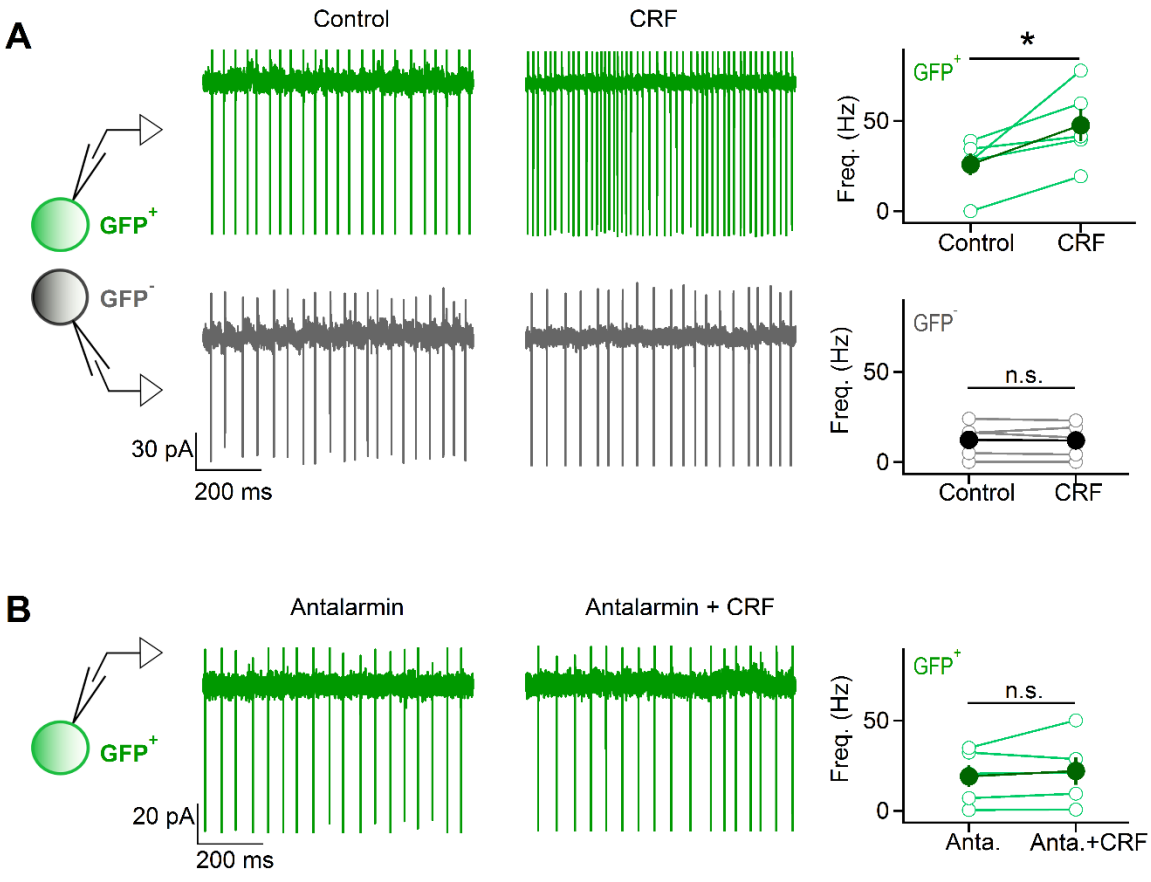
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Figure 7. CRF increases firing rates in CRFR1 neurons. (A) Left, Simultaneous loose-patch voltage clamp recordings from neighboring GFP+ (green traces) and GFP- (grey traces) neurons (<100 μm intersomatic distance). Bath application of 30 nM CRF led to an increase in firing in the GFP+ neuron, but not in the GFP- neuron. Right, Quantification of CRF-induced changes in firing rates of GFP+ (top) and GFP- neurons (bottom). All data were recorded from mixed pairs (n=5 pairs). (B) Representative loose-patch recording from a GFP+ neuron in the presence of 1μM Antalarmin in the bath solution. Bath application of 30 nM CRF did not lead to an increase in firing frequency. Right, Quantification of CRF-induced changes in firing rates of GFP+ neurons recorded in the presence of Antalarmin. n=5 neurons. Note: These experiments and creation of the figure were done in collaboration with Rajan Dasgupta and Michael Beierlein, PhD who have provided permission to use the figure.

Conclusions

These experiments provided the necessary data to illustrate that GFP fluorescence in CRFR1-GFP mice faithfully reports CRFR1 expression. In our experimental paradigm, we recorded from 5 pairs of GFP+ and GFP- neurons while performing bath application of CRF. We observed an increase in firing in response to CRF in all 5 GFP+ neurons, and the absence of a response in all 5 of the GFP- neurons. Using the CRFR1-specific antagonist, we also verified that the observed increase in the mean firing rate of GFP+ CRFR1 expressing neurons was mediated through CRFR1. In sum, the CRFR1-GFP mouse is a viable, reliable tool to visualize CRFR1 expression until a dependable and cost effective antibody is produced.

Note: These experiments were performed in collaboration with the Beierlein Lab, McGovern Medical School, Houston, TX, USA

CRFR1 Expressing Neurons in the GPe are Prototypic

In Brief

The GPe has recently been shown to contain highly heterogeneous cell types that arise from distinct neural lineages and have differing afferent and efferent connections (Abdi et al., 2015; Dodson et al., 2015; Glajch et al., 2016; Hegeman et al., 2016; Hernández et al., 2015; Mallet et al., 2012; Saunders et al., 2016). Electrophysiological recordings in awake macaque provided the first insights into this diversity. Two classes of neurons were identified: “high-frequency pausers” and “low-frequency bursters”. One class of neuron fired regularly at high-frequency with brief periods of silence, while the second class remained relatively silent but had the capability to fire at high-frequency for brief periods of time (DeLong, 1971). Due to the highly dynamic electrophysiological properties of GPe neurons, further refinement of neuronal subtypes necessitates discriminating the neuronal types based on mRNA expression profiles.

Based on firing patterns, two terms have been assigned to the neuronal classes Delong discovered: prototypic and arkypallidal. Prototypic neurons most closely correspond to the “high-frequency pausers,” and arkypallidal neurons are analogous to the previously observed “low-frequency bursters” (DeLong, 1971; Mallet et al., 2012). These neurons can be discriminated by their expression of molecular markers and major projection sites. Prototypic neurons are derived from the medial ganglionic eminence and primarily project to the subthalamic nucleus (STN). They uniquely express the transcription factor Nkx2.1 and/or the calcium binding protein Parvalbumin (PV). Arkypallidal neurons are derived from the lateral ganglionic eminence and are defined by their expression of the transcription factor FoxP2. All (>99%) FoxP2

neurons co-express the transcription factor Npas1. Arkypallidal neurons that express Npas1 project exclusively to the STR (Abdi et al., 2015; Flandin et al., 2010; Mallet et al., 2012; Nóbrega-Pereira et al., 2010). The robust expression of CRFR1 in the GPe raises the intriguing question of whether CRFR1 expression is restricted to a particular neuronal subtype (Justice et al., 2008; Sztainberg et al., 2011). Moreover, why is the CRF receptor, which partly mediates anxiogenesis, abundantly expressed in the GPe? Activation of one neuronal subtype in the GPe by CRF/CRFR1 signaling may provide insight into the role of the BG in stress and stress-related behaviors. To determine whether CRFR1 expression is restricted to one subtype or is ubiquitously expressed in the GPe, I performed immunofluorescent labeling for prototypic and arkypallidal neuronal markers in BAC transgenic CRFR1-GFP mice. In CRFR1-GFP mice, it has been validated that GFP expresses in the same pattern as CRFR1 in the GPe (Justice et al., 2008; Sztainberg et al., 2011). Using an anti-GFP primary antibody as a proxy for CRFR1 expression, I examined co-expression of CRFR1 with prototypic and arkypallidal neuronal markers to determine which subtypes of neurons express CRFR1 by immunofluorescence. My technique allowed me to reveal that CRFR1 is expressed exclusively by prototypic neurons.

Results

I performed immunofluorescent labeling in CRFR1-GFP mice to systematically characterize the prototypic or arkypallidal identity of CRFR1 neurons in the GPe. I used the neuronal marker NeuN to determine that CRFR1 is expressed by 61.1 ± 4.72% of GPe neurons (Fig.8 A-C) (Fields et al., 2015). 86.0 ± 5.18% of CRFR1-GFP neurons co-express the prototypic neuron marker Nkx2.1, while 72.6 ± 0.93% of Nkx2.1 neurons co-express GFP (Fig.9 A-C) (Mallet et al., 2012). I observed that 53.3 ± 3.20% of the CRFR1-GFP neurons in the GPe express PV consistent with previous reports (Fig.9 D-F) (Sztainberg et al., 2011). Of great interest, nearly all PV neurons (98.6 ± 0.44%) express CRFR1-GFP (Fig.9 N). A subpopulation of Nkx2.1+ GPe neurons co-expresses PV (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015). My results indicated that 36.3% ± 2.35% of CRFR1-GFP+ neurons express both Nkx2.1 and PV (Fig.10 A-D). I found that no CRFR1-GFP neurons express the arkypallidal neuronal marker FoxP2 (Fig.9 J-L) (Abdi et al., 2015; Dodson et al., 2015). However, a small population of CRFR1-GFP+ neurons expresses Npas1 (6.49% ± 1.10%), while roughly 10% of Npas1+ neurons co-express CRFR1-GFP (Fig.9 G-I).

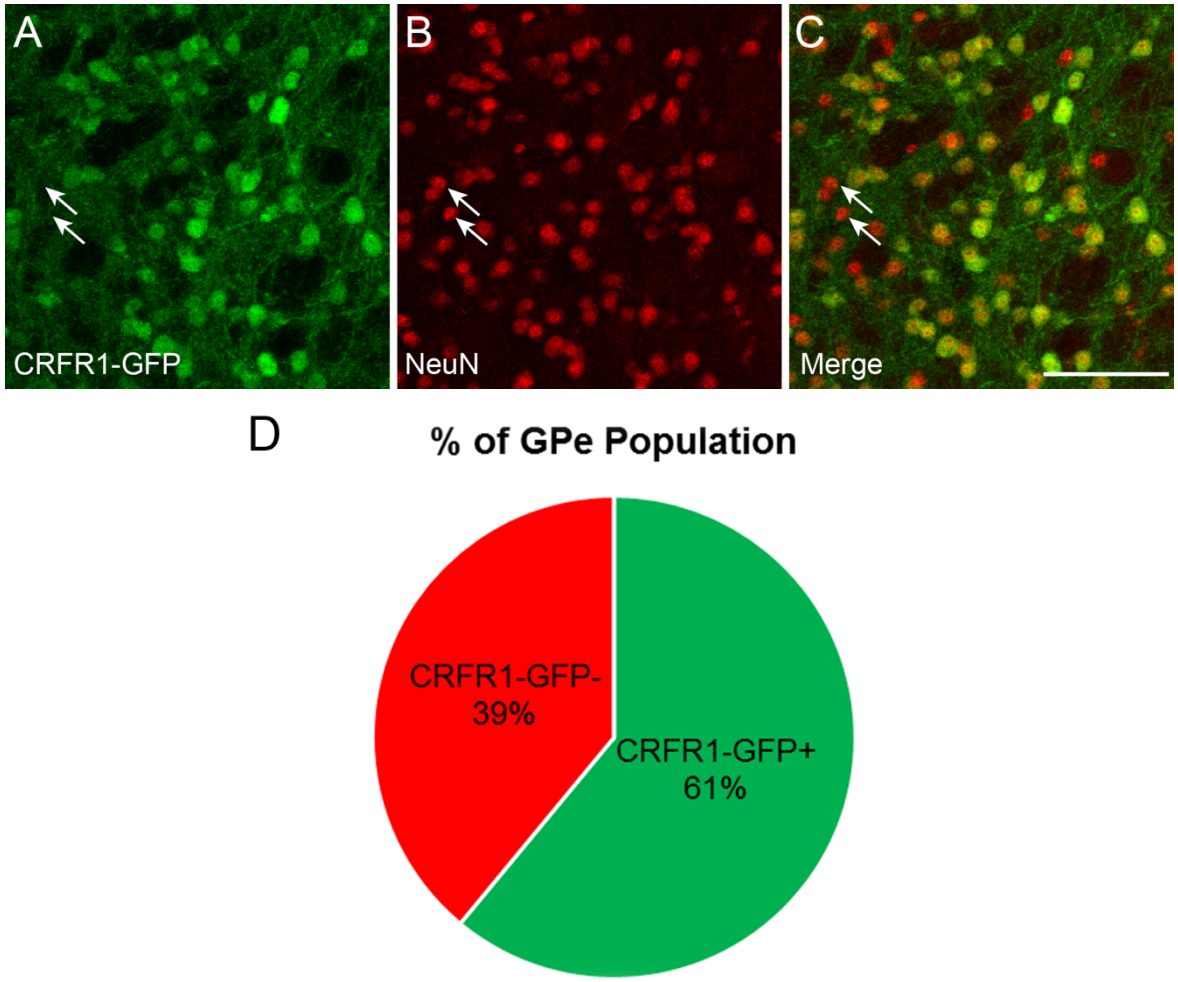
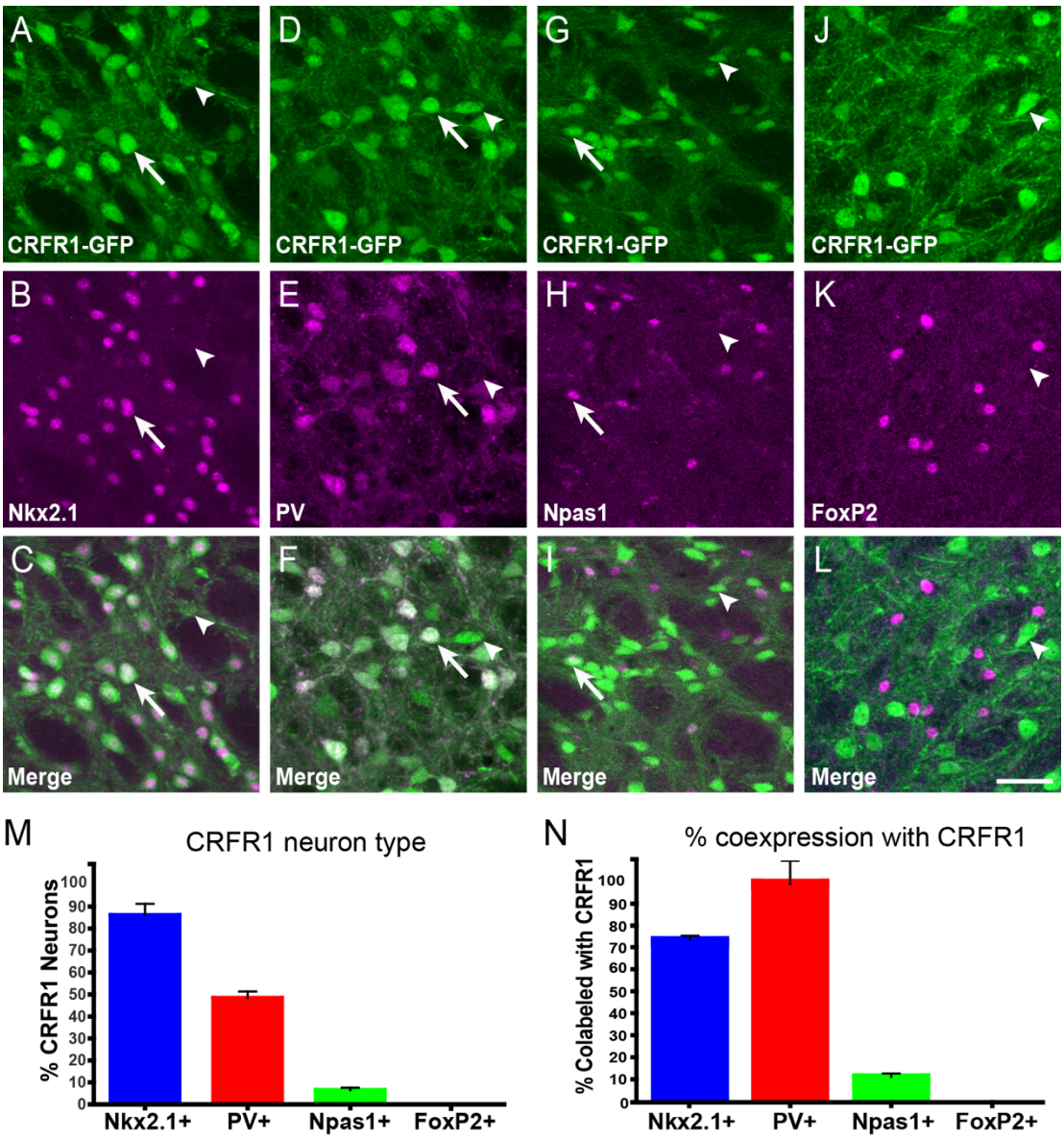


Figure 8. Quantification of CRFR1-GFP in the GPe. (A) CRFR1-GFP expression in the GPe. (B) Immunofluorescent labeling of the neuronal marker NeuN. (C) Merged image showing the overlay between CRFR1-GFP and NeuN. The arrows indicate neurons that do not express CRFR1-GFP. Scale Bar = 100 μm (D) 61% of the GPe expresses CRFR1-GFP while 39% does not.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Nkx2.1
E
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Npas1
K
FoxP2
C
Merge
F
Merge
I
Merge
L
Merge
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CRFR1 neuron type
% CRFR1 Neurons
100
90
80
70
60
50
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0
Nkx2.1+
PV+
Npas1+
FoxP2+
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% coexpression with CRFR1
% Colabeled with CRFR1
100
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80
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Nkx2.1+
PV+
Npas1+
FoxP2+

Figure 9. Molecular characterization of CRFR1+ neurons in the GPe. CRFR1-GFP brain sections were stained for Nkx2.1 (A-C), PV (D-F), Npas1 (G-I), and FoxP2 (J-L). The majority of CRFR1 positive GPe neurons are also positive for Nkx2.1 and/or PV. A small number of CRFR1 cells are Npas1+ (G-I), and no FoxP2 neurons express CRFR1 (J-L). (M) Quantification of the percent overlap of each molecular marker with GFP. (N) Quantification of the percent of each defined cell type that co-expresses CRFR1-GFP. Arrows indicate examples of double labeled cells. Arrowheads indicate examples of singly labeled cells. Scale bar = 50 μm.

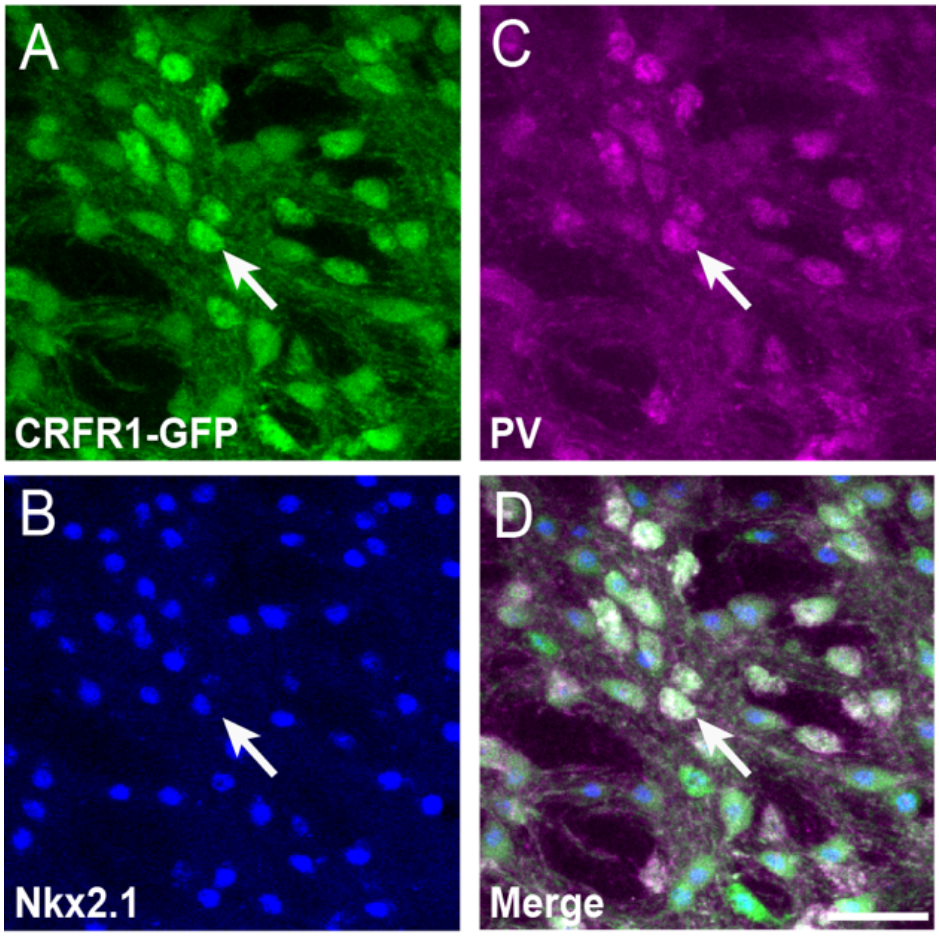


Figure 10. Co-expression of Nkx2.1 and PV in CRFR1-GFP cells. (A) CRFR1-GFP expressing neurons in the GPe. (B) Immunofluorescent labeling of Nkx2.1. (C) Immunofluorescent labeling of PV in the GPe. (D) Merged image showing that Nkx2.1 and PV are co-expressed in some CRFR1-GFP cells. Scale Bar = 50 μm.

Conclusions

Although many antibodies to CRFR1 have been prepared and used by various groups, none have proved reliable (Justice et al., 2008). The BAC transgenic CRFR1-GFP mouse (see Experimental Procedures) became a necessity to provide a proxy for CRFR1 expression through GFP fluorescence. Immunohistochemical data obtained by these experiments provide evidence that CRFR1 is expressed exclusively by prototypic neurons (Nkx2.1 and/or PV), and is absent from arkypallidal neurons (FoxP2). I observed a small proportion of CRFR1-GFP neurons that were positive for Npas1. All (>99%) FoxP2+ neurons are arkypallidal neurons and express Npas1, however Npas1 co-expression with Nkx2.1 and PV has been observed (Abdi et al., 2015; Hernández et al., 2015). This fact leads one to draw the conclusion that Npas1 neurons are arkypallidal. However, a distinction exists between Npas1+ neurons that co-express the prototypic neuronal markers Nkx2.1 and/or PV and Npas1+ neurons that co-express the arkypallidal neuronal marker FoxP2. Therefore, Npas1 cannot be used to neatly discriminate between prototypic and arkypallidal neurons (Dodson et al., 2015). My data indicates that CRFR1-GFP does not co-express with FoxP2, therefore the CRFR1-GFP+/Npas1+ neurons I observed are prototypic further bolstering my conclusion that CRFR1 is expressed exclusively by prototypic neurons (Abdi et al., 2015; Hernández et al., 2015). Although Npas1 cannot be used to discriminate between prototypic and arkypallidal neurons, Npas1 is an excellent marker of neurons that form the pallidostriatal pathway (Abdi et al., 2015; Glajch et al., 2016). My observation that CRFR1 is expressed by prototypic neurons that primarily make up the pallidosubthalamic tract suggests that the small proportion of

STR targeting CRFR1-GFP+/Npas1+ neurons allows CRF/CRFR1 signaling to have distinct and disparate functions based on activation of GPe neuronal subtypes.

It is well documented that the GPe is involved in processing sensorimotor information and recent literature has provided evidence that the GPe projects to all basal ganglia nuclei and is not an isolated member of the motor inhibiting indirect pathway (DeLong, 1971; Glajch et al., 2016; Mallet et al., 2012; Obeso et al., 2006). The primary input to the GPe is the striatum (STR) and STR neurons are known as medium spiny neurons (MSNs). Output of the STR can be divided into two main projections that have opposing effects on movement, the direct and indirect pathways (DeLong, 1990). Direct pathway MSNs express the dopamine excitatory receptor D1 and project primarily to the pars reticulata of the substantia nigra (SNr). In addition, these neurons send input to the internal globus pallidus (GPi), known as the entopeduncular nucleus (EP) in mouse. Indirect pathway MSNs express the dopamine inhibitory D2 receptor and send their projections to the GPe. Not only are these pathways differentiated based on the expression of dopamine receptor subtypes and projection sites, but they have opposing effects on movement, reinforcement, and reward-related behavior (Ferguson et al., 2011; Kravitz et al., 2012; Lobo et al., 2010; Wall et al., 2013).

The GPe mimics the organization of the STR with two neuronal classes that express CRFR1 in a distinct pattern and have different projection sites, similar to the direct D1 and indirect D2 pathways in the STR. It is reasonable to draw a parallel between the STR and the GPe. The GPe's two neuronal pathways, the prototypic pallidosubthalamic pathway and the arkypallidal/Npas1+ pallidostriatal pathway may

serve to have opposing roles in various behaviors similar to the STR. All prototypic neurons innervate at least one of the “downstream” BG nuclei, primarily the STN. Arkypallidal neurons only innervate the STR.

As individual GPe neurons display heterogeneity in the firing frequency and regularity of their autonomous firing, long duration recordings have provided evidence that individual neurons, regardless of identity, can fluctuate or “wander” their firing frequency over a few minutes or spontaneously (Deister et al., 2013). In fact, the rate and pattern of individual neuron firing may sample all of the properties observed in the variable GPe population in totality. These findings emphasize the difficulty in discriminating prototypic and arkypallidal neurons by electrophysiological properties alone and that advances have been made by identifying molecular markers to discriminate prototypic from arkypallidal neurons (Deister et al., 2013).

Very recently, it has been described that arkypallidal and prototypic neurons can be distinguished based on their activity across the sleep-wake cycle. This adds to the available techniques to distinguish between the two neuronal subtypes.

Specifically, arkypallidal neurons have a distinct function from prototytpic neurons as revealed by electrophysiological recording from “sleep-wake cycle identified” arkypallidal neurons in awake rats. In addition to supporting the evidence that the GPe contains two distinct cell types, these findings demonstrate that the GPe may play a role in arousal state.

The Stop task utilizes a chamber in which a rat maintains its nose in a central port. In this task, a tone, or “Go” signal, instructs the rat to move its head to the left or right. A white noise burst serves as the “Stop” signal that signals the mouse to stop

the movement and fixate back in the central port. By screening neurons that changed firing rates shortly after the stop signal, evidence was provided that arkypallidal neurons provide a “Stop” signal by their GABAergic projections to the STR. The arkypallidal “Stop” signal cancels a “Go” signal which is sent by the STR to the SNr resulting in the ending of the imminent movement (Mallet et al., 2016). In addition to the cancellation of imminent movement, current theories of the indirect pathway (motor inhibiting) “Stop” function are that it serves to restrain unrewarded actions.

As part of an integrated stress response, CRF is released. CRFR1 is expressed by very few Npas1 neurons, which are primarily arkypallidal and all send projections to the STR. If CRF is released in the GPe, I speculate that arkypallidal neurons would not “Stop” the stress response to promote survival. Prototypic neurons would send a “Go” signal, an action that promotes survival, in direct opposition of arkypallidal to STR stop signals, but this remains to be proven (Collins and Frank, 2014).

In 6-OHDA lesioned rat models of Parkinson’s disease (PD), chronic dopamine depletion greatly decreases the firing of Npas1-STR neurons. PV+/prototypic neuron firing properties are not impacted by dopamine loss (Hernandez et al., 2015). As a result of dopamine loss, aberrant Npas1 neuron firing is likely caused by higher inhibitory tone. Inhibition from the STR becomes stronger in the absence of dopamine because the inhibitory D2 receptor is not activated. This would reduce the amount of GABA being released in the GPe allowing this indirect motor inhibiting pathway nucleus to fire more frequently. This suggests that input from the STR to the different neuronal subtypes of the GPe is not equal (Abdi et al., 2015). Of significance, I have shown that CRFR1 is expressed by prototypic neurons. The question is raised

whether a decrease in activity of Npas1 neurons in the dopamine depleted brain is further exacerbated by CRF signaling to CRFR1 prototypic neurons or if CRF signaling is modulated to counterbalance this abnormality.

Determining the role of CRF/CRFR1 signaling in prototypic neurons becomes crucial if I want to provide evidence to whether or how CRF plays a role in Parkinson's symptoms. With evidence that the two GPe neuronal subtypes receive differential inputs, their downstream targets must similarly be affected leading to aberrant functioning in other key basal ganglia nuclei that have the potential to be exacerbated by CRF/CRFR1 signaling.

CRF Neurons Project to and Make Synapses in the External Globus Pallidus

In Brief

Previous experiments detecting CRF expression in the brain revealed that CRF+ fibers exist in the GPe of rat (Merchenthaler, 1984). The GPe expresses high levels of CRFR1; however, it is not considered a primary release site of CRF as only a single study has addressed the possible role of CRFR1 in anxiety-related behavior mediated by the GPe (Justice et al., 2008; Sztainberg et al., 2011; Van Pett et al., 2000). The elusive question that remains is the source of CRF input to the GPe.

CRF neurons are highly concentrated in stress-responsive nuclei including the paraventricular nucleus of the hypothalamus (PVN), central nucleus of the amygdala (CeA) [(lateral (CeAl) and medial (CeAm)] and lateral dorsal subdivision of the bed nucleus of stria terminalis (BSTld) (Sawchenko et al., 1993). To determine if one nucleus preferentially projected to the GPe, I performed experiments to examine CRF neuronal projections to the GPe in mouse using immunohistochemistry in the triple transgenic mouse, CRFR1-GFP; CRF-cre; floxed-stop ^{td}tomato (Ai9). These mice express GFP in neurons that express CRFR1 and the fluorescent reporter ^{td}tomato in CRF neurons and fibers. To determine the origin of CRF input to the GPe, I utilized rabies virus mediated retrograde tracing in the novel BAC transgenic line CRFR1-cre which express Cre in neurons that express CRFR1 and found that CRF-rich stress regions make synapses on neurons that express CRFR1. As I observed a small population of Npas1+ neurons express CRFR1, I performed rabies tracing in Npas1-cre mice as well. Npas1-cre mice express Cre in neurons that express Npas1 (Hernández et al., 2015). Intriguingly, as Npas1 neurons that express CRFR1 are few

in number, I found that the same stress regions that project to CRFR1 expressing neurons project to Npas1 neurons as well.

For the retrograde tracing experiments with rabies virus, I made bilateral stereotaxic injections of Cre-dependent TVA-mCherry/G encoding AAVs in the GPe of CRFR1-cre BAC and Npas1-cre BAC transgenic mice. Neurons successfully expressing the avian receptor TVA express mCherry in a distinguishable punctate fashion. The two gene cassettes, TVA and G, are required for the subsequent infection and transmission of the rabies virus (Fig.12 A). Two weeks later, I injected the same coordinates with pseudotyped rabies virus (PTRV) that expresses GFP (EnvA-SADΔG-GFP). This modified rabies virus has had its coat protein, G, replaced with GFP (Fig.12 B). The virus is pseudotyped with the avian coat protein EnvA, which limits viral infection to neurons that express the avian receptor TVA. In the presence of G, also delivered in the first injection, the rabies virus can package and jump retrograde one synapse. Transmission is limited to one synapse because the newly infected neuron does not express G, and subsequently, the rabies virus cannot package and continue its retrograde “hop” (Wall et al., 2013; Watabe-Uchida et al., 2012; Wickersham et al., 2007). This technique generally produced 5-10 “starter” neurons (neurons competent to generate rabies virus capable of retrograde transmission). “Starter” neurons are distinguished by their co-expression of TVA-mCherry fluorescence and PTRV-GFP (Fig.12 C). Neurons that only express GFP are “traced” neurons which project to and make monosynaptic connections with “starter” neurons which are only located in the GPe as controlled by highly precise stereotaxic injection. Using this array of highly technical genetic tools and transgenic mice, I have

been the first to identify and map the source of synaptic input from CRF neurons to the GPe.

Results

To investigate CRF neuron projections to the GPe, I imaged sections of the GPe from CRFR1-GFP; CRF-cre; floxed-stop ^{td}tomato (Ai9) mice. CRF-IRES-Cre mice have been shown to express Cre accurately in neurons that express CRF (Chen et al., 2015; Taniguchi et al., 2011). In sections of the GPe, in mice carrying all three transgenes, CRFR1 neurons are green fluorescent and projections from CRF neurons are red fluorescent. ^{td}tomato fibers from CRF neurons can be seen projecting within the GPe. The origin of these CRF neuron fibers remains unknown. Co-labeling for the presynaptic marker Synapsin-1 reveals presynaptic terminals between ^{td}tomato+ axonal fibers and CRFR1-GFP+ dendrites and cell bodies (Fig.11 A-D), identified visually.

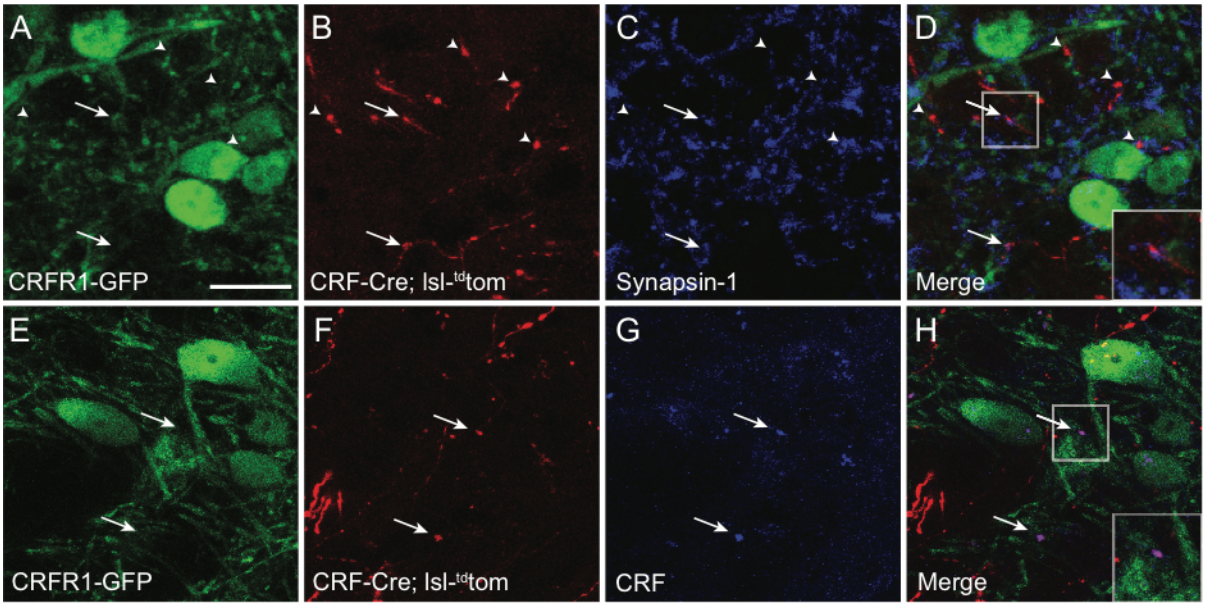


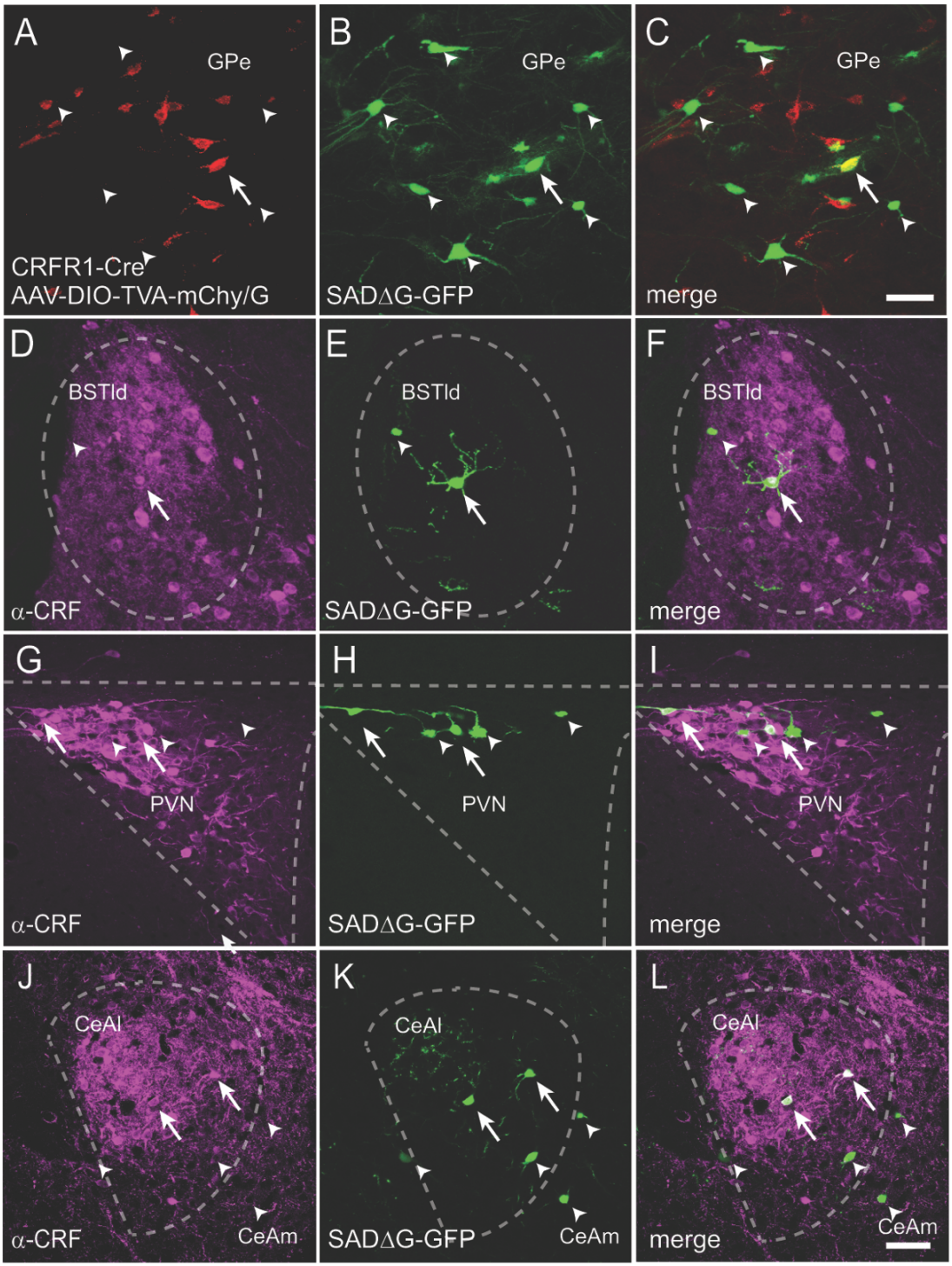
Figure 11. CRF neurons have presynaptic terminals and CRF peptide located in the GPe. Images of the GPe from mice carrying CRFR1-GFP, CRF-cre and a Cre dependent ^{td}tomato reporter (lsl-^{td}tom) reveal projections from CRF neurons in close proximity to CRFR1-GFP+ neurons. (A-D) Staining of sections for Synapsin-1 identifies synaptic puncta where the projections meet (arrows), as well as puncta that are Synapsin-1 negative (arrowheads). (E-H) Staining for CRF peptide shows that many of the larger puncta contain CRF peptide in the GPe. Scale bar = 25 μm

When I stained for CRF peptide, CRF is located in ^{td}tomato+ axonal puncta in the GPe, adjacent to CRFR1-GFP+ dendritic fibers (Fig.11 E-H). The evidence provided by these experiments indicates that presynaptic terminals of CRF neurons exist in the GPe which possibly release CRF onto cell bodies and dendrites of CRFR1-GFP+ neurons. These experiments did not definitively confirm that CRF is released at these terminals. In addition, staining for Synapsin-1 in the absence of co-staining with a postsynaptic marker does not allow for the conclusion that synapses between CRF neuron fibers and CRFR1-GFP neurons are present. These experiments demonstrate that (1) CRF neurons project to the GPe, (2) CRF is present in CRF neuronal fibers in the GPe, and (3) CRF fibers have presynaptic terminals in close proximity to CRFR1 expressing neurons.

To provide evidence that CRF neurons make synapses on CRFR1 neurons, I utilized the PTRV technique to reveal the synapse formation between CRFR1 “starter” neurons and CRF-positive “traced” neurons. To identify CRF neurons, I made ICV injections of colchicine to stop vesicular transport into mice that had undergone the PTRV procedure to allow for staining of CRF neurons. As CRF is a neuropeptide, it is located in vesicles throughout the axon. When staining for CRF followed by a secondary fluorophore, the resulting fluorescence will appear in fields instead of single neurons. By stopping vesicular transport, CRF vesicles remain in the soma. This allows immunohistochemistry to identify individual neurons that contain the neuropeptide (Wang et al., 2011).

Using CRFR1-cre mice, this is the first study to provide evidence not only from where CRF input to the GPe originates but that it originates in key stress nuclei; the BSTld (Fig.12 D-F), PVN (Fig.12 G-I), and CeAl (Fig.12 J-L). When I stained for CRF in Npas1-cre mice, I found that the same nuclei; the BSTld (Fig.13 A-C), PVN (Fig.13 D-F), and CeAl (Fig.13 G-I), project to Npas1 neurons. The CRF neurons most likely project to the small proportion of Npas1 neurons that express CRFR1. I calculated a connectivity ratio of the observed neurons in the stress regions. The connectivity ratio is defined as the number of "traced" neurons in each nucleus per "starter" neurons in the GPe. I found that the stress region connectivity ratio in CRFR1-cre and Npas1-cre is distinct indicating differential patterns of innervation. Npas1 neurons have a significantly higher connectivity ratio from both the CeAl (2.23 ± 0.33 vs. 0.97 ± 0.27; $t_{(9)}$=2.87, p<0.05) and CeAm (0.88 ± 0.34 vs. 0.08 ± 0.04; $t_{(6)}$=3.18, p<0.01) compared to the stress region connectivity ratio in CRFR1-cre mice. Tracing in CRFR1-cre mice revealed that the PVN has a higher connectivity ratio (0.47 ± 0.26 vs. 0.14 ± 0.08; $t_{(8)}$=1.22, p=0.25) compared to Npas1-cre, although this did not reach statistical significance likely due to variability in injections. The connectivity ratio from the BSTld did not differ (Fig.14 A). It is intriguing that CRFR1+ neurons and Npas1+ neurons receive differential input from the stress regions. This suggests that possibly the stress regions activate the neurons at different times or that they may have disparate functions. For example, the activation of CRFR1+ neurons by the PVN may occur during the activation of the stress response as CRF neurons release CRF at the initiation of the stress response. It is possible that the CeA may preferentially target

Npas1+ neurons at the termination of the stress response but this remains to be explored.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BSTld
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F
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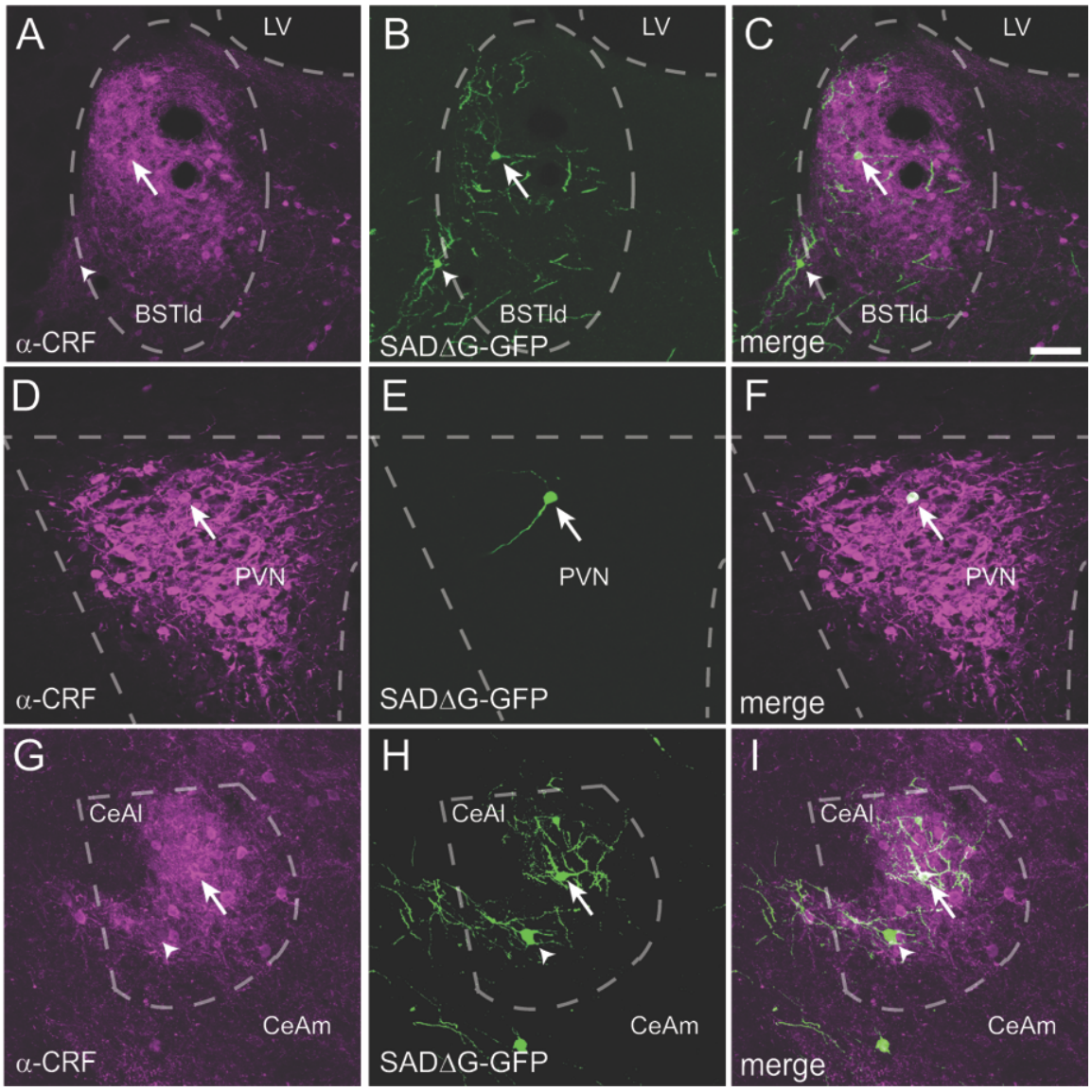
Figure 12. Rabies viral tracing of monosynaptic inputs to GPe CRFR1+ neurons. (A) Injection of AAV helper viruses encoding Cre-dependent TVA-mCherry (red) and G allow for infection by EnvA pseudotyped, G-deleted rabies virus that expresses GFP (B, SADΔG-GFP, green). (C) The merged image shows a "starter" neuron that expresses both the helper and the rabies viruses (yellow, arrow) surrounded by neurons expressing GFP only (green, arrowheads) which make synapses on a "starter" neuron. In mice traced from CRFR1+ neurons in the GPe, I found connected neurons in the BSTId (D-F), the PVN (G-I) and the CeAl (J-L). Staining of sections of these nuclei show that some of these neurons contain CRF peptide (magenta, arrows), while other neurons do not (arrowheads). A-C; D-L = 50 μM.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CeAm
merge

Figure 13. CRF neurons project to Npas1+ GPe neurons. CRF staining of sections from Npas1-cre tracing experiments. I found CRF neurons (magenta) in the BSTId (A-C), PVN (D-F), and CeA (G-I) that were traced from Npas1+ neurons in the GPe (green). Arrows indicate “traced” neurons that are positive for CRF peptide, arrowheads indicate “traced” neurons that are CRF negative. Scale bar = 50 μm. CeAl = lateral division of the central nucleus of the amygdala. CeAm = medial division of the central nucleus of the amygdala.

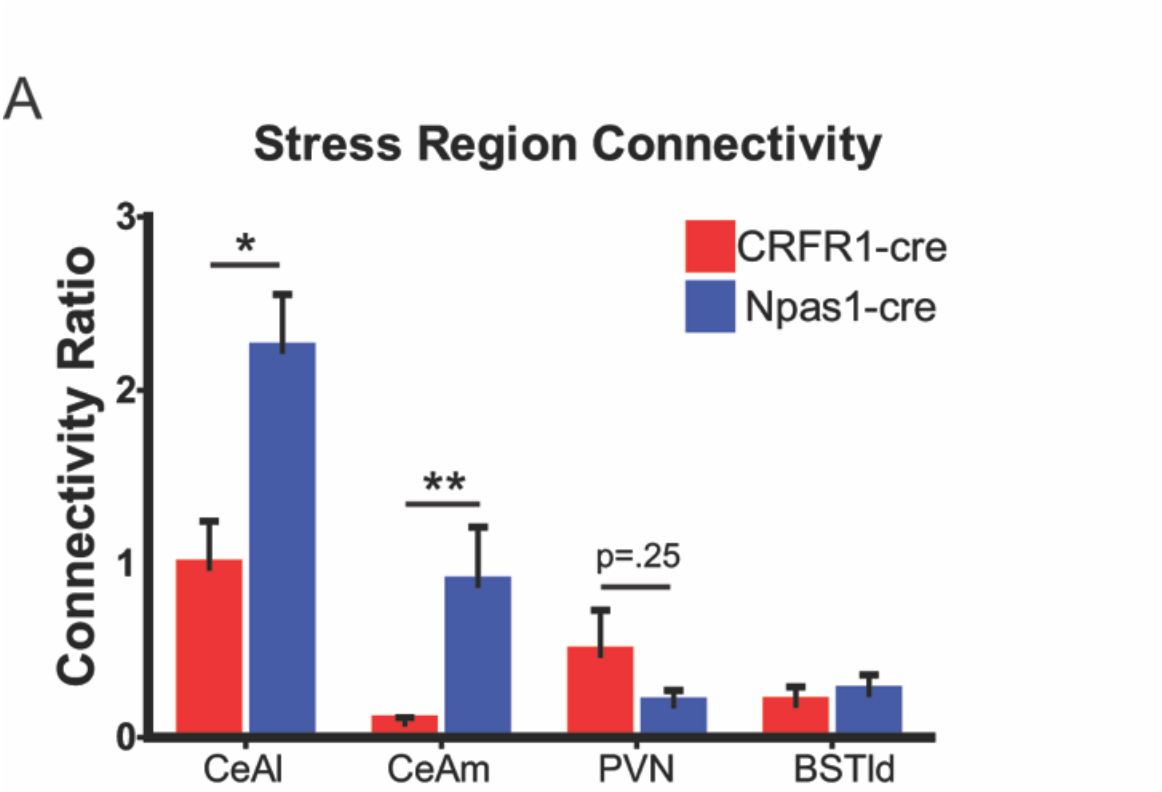


Figure 14. Quantification of the connectivity between key stress responsive nuclei and CRFR1 neurons (red) and Npas1 neurons (blue) in the GPe. (A) The CeAl and CeAm make more connections with Npas1+ neurons than with CRFR1+ neurons. * = $p<0.05$; ** = $p<0.01$, n=3 per transgenic mouse line. Connectivity ratio is the number of "traced" neurons present in each nucleus per "starter" neuron in the GPe.

Conclusions

These experiments provide evidence of the origin of CRF input to the GPe, which was previously unknown. I provide novel evidence that the source of this input is from key stress regions of the brain. Tracing experiments in CRFR1-cre mice indicate that the BSTld, CeA, and PVN send projections and synapse onto CRFR1 neurons in the GPe. While this has never been shown before, it was not completely unexpected because these regions express CRF abundantly. What was unexpected is that the same regions project to Npas1 neurons in the GPe. Presumably, it is the small population (<7%) of CRFR1 neurons that express Npas1 that receive projections from CRF neurons in stress regions. The question is raised as to why the Npas1+/CRFR1+ neurons receive projections from the same regions that project to exclusively CRFR1 expressing neurons. Performing the tracing study in both CRFR1-cre and Npas1-cre lines raises the possibility that all prototypic neurons receive CRF synapses from stress regions. Interestingly, as discussed previously, prototypic neurons form the pallidosubthalamic tract while Npas1 neurons project exclusively to the STR. This implies that there may be different functions of CRF in the GPe. A small minority of CRFR1 expressing Npas1 neurons projecting to the STR may allow CRF to bias striatal functioning and output. The primary input to the GPe is the STR. CRFR1 receptors on Npas1 neurons would allow for CRF/CRFR1 signaling to provide feedback from GPe activity to the STR. The GPe is a GABAergic nucleus and sends inhibitory signals primarily to the STN (DeLong and Wichmann, 2007). As part of the GPe, Npas1 neurons are also GABAergic and transmit inhibitory tone to the STR. This CRF-activated pallidostriatal pathway may create an inhibition of inhibition loop.

Depending on the MSNs that Npas1 neurons project to, which is yet to be determined, CRF activation of Npas1+/CRFR1+ neurons may increase activity in the GPe by inhibiting the inhibition feeding into the GPe from the STR. In addition, CRF activation of the canonical prototypic neurons may act synergistically to allow CRF/CRFR1 signaling to activate the GPe while simultaneously decreasing inhibitory input from the STR.

The only glutamatergic, excitatory nucleus in the BG is the STN projection to the GPi (DeLong and Wichmann, 2007). Based on recent literature that proposes the GPe projects to all nuclei in the BG, it becomes apparent that an additional source of excitatory neuron activating neuropeptide such as CRF could have a dramatic effect on the function of many regions in the BG. Another point of interest is that tracing done in CRFR1-cre mice indicates that there are more projections from the PVN to the CRFR1 GPe population while there are more projections from the CeAl and CeAm nuclei to GPe Npas1 neurons. PVN CRF neurons are able to co-release excitatory glutamate, while CeA CRF neurons co-release inhibitory GABA (Füzesi et al., 2016; Partridge et al., 2016). PVN CRF neurons may communicate with CRFR1 expressing GPe neurons first through fast glutamatergic synapses to activate neurons followed by CRF to maintain their activation at the initiation of a stress response. The CeA may dampen the effects of CRF on Npas1 neurons by co-release of GABA restoring inhibition from the STR possibly at the termination of a stress response.

In summary, this study is the first to provide evidence that CRF neurons in key limbic and autonomic stress regions through long-range projections form synaptic

connections with GPe neurons indicating that the GPe and basal ganglia may play an important role in the stress response not yet characterized.

Interconnectivity of Prototypic and Arkypallidal Neurons in the GPe

In Brief

In the last chapter, I utilized PTRV tracing techniques to identify novel long-range CRF projections between key stress regions and CRFR1+ or Npas1+ neurons in the GPe. While quantifying the number of “traced” neurons in sections from the entire brain, I uncovered that the GPe contains complex microcircuitry. To evaluate local GPe connectivity, I utilized PTRV retrograde tracing in both CRFR1-cre and Npas1-cre mice and immunohistochemistry with specific markers to neuronal subtypes in the GPe. In the interconnected GPe, I found that CRFR1+ prototypic neurons only receive synaptic input from other prototypic neurons. This finding may be extrapolated to propose the hypothesis that only prototypic neurons synapse on other prototypic neurons within the GPe.

Results

As part of my analysis of PTRV mice, I calculated a connectivity ratio for the major regions projecting to the GPe. Once I completed the necessary injections for the PTRV technique and sacrificed the mice, I prepared brain sections from the entire brain. I visually inspected the sections for neurons expressing GFP, "traced" neurons. I quantified the number of "traced" neurons in numerous brain regions known to send projections to the GPe or BG. Also, I included the hypothalamus and amygdala due to their close proximity to the GPe. Once I quantified the number of "traced" neurons throughout the brain, I quantified the number of "starter" neurons in the GPe and calculated a connectivity ratio for each region the same way I calculated the connectivity ratio for stress regions. The STR is known to be the primary source of input to the GPe, however I found that the nucleus with the second highest input to the GPe was the GPe itself, manifesting almost the same connectivity ratio as that of the STR. For example, the ratio between "traced" striatal neurons to "starter" GPe neurons is ~12 to 1. Likewise, the ratio between "traced" CRFR1+ GPe neurons to "starter" GPe CRFR1+ neurons is ~10 to 1 (Fig.15 A). This provides support for an argument that the GPe is highly interconnected and forms an inhibitory network as the GPe is GABAergic.

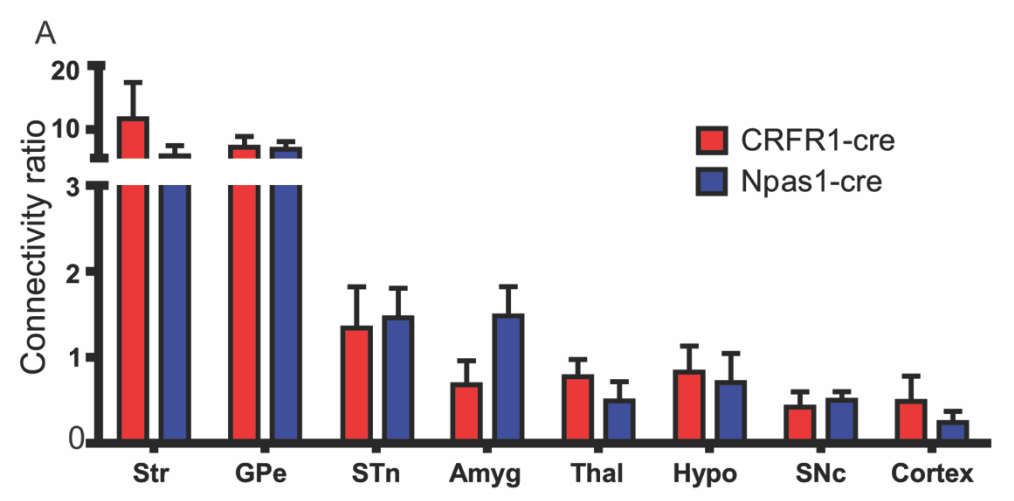


Figure 15. Quantification of CRFR1-cre (red) and Npas1-cre (blue) monosynaptic tracing experiments expressed as a Connectivity Ratio. (A) Connectivity ratio is the number of traced neurons in each region divided by the number of starter neurons in that experiment. n=3 mice per line. Str = Striatum, GPe = external globus pallidus, STn = Subthalamic Nucleus, Amyg = Amygdala, Thal = Thalamus, Hypo = Hypothalamus, SNc = Substantia Nigra pars compacta.

In CRFR1-cre mice, I observed “starter” neurons that were positive for the prototypic markers Nkx2.1 (Fig.16 A) and PV (Fig.16 B). Also, I observed “starter” neurons that were positive for Npas1 (Fig.16 C), suggesting that these neurons represent the small population of CRFR1+/Npas1+ prototypic neurons. I did not observe any CRFR1+ “starter” neurons that were positive for the arkypallidal marker FoxP2 (Fig.16 D). These results are consistent with my findings from the experiments using the CRFR1-GFP mice as described previously.

In Npas1-cre mice, I observed “starter” neurons that were positive for either Nkx2.1 (Fig.17 A), Npas1 (Fig.17 C), or FoxP2 (Fig.17 D). I did not observe any “starter” neurons that were positive for PV as Npas1 and PV neurons are a non-overlapping class (Fig.17 C) (Hernández et al., 2015). While some Npas1-cre mice had “starter” neurons that were positive for Nkx2.1, suggesting that these are prototypic neurons; overwhelmingly, Npas1-cre “starter” neurons co-express the arkypallidal marker FoxP2. These results indicate that “starter” neurons generated in CRFR1-cre are prototypic while “starter” neurons generated in Npas1-cre are pallidostriatal projecting, as they are Npas1+. Additionally, the majority have a high probability of having arkypallidal neuronal identity (FoxP2). In the case of Npas1, combining the visibility of Cre through mCherry expression and neuron specific markers, I was able to be confident that Npas1-cre expresses Cre in the pattern I expect based on previous findings from other groups. Other groups have likewise demonstrated that co-expression of Npas1 with Nkx2.1 or FoxP2 exists (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015). This confirms that any future

experiments using these Cre lines are reliable because Cre is expressed in the correct neurons.

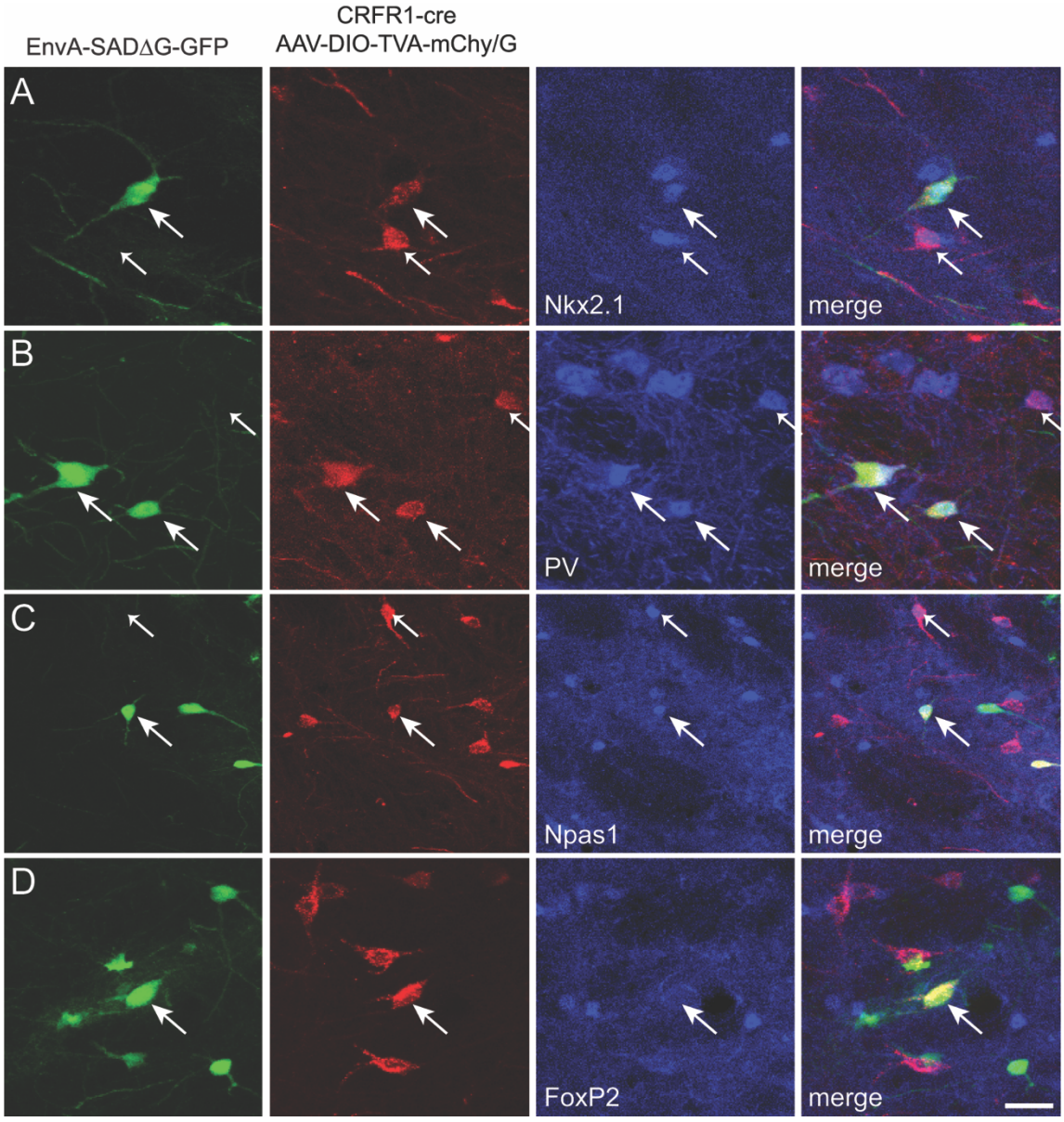

EnvA-SADΔG-GFP
CRFR1-cre
AAV-DIO-TVA-mChy/G
A
Nkx2.1
merge
B
PV
merge
C
Npas1
merge
D
FoxP2
merge

Figure 16. Characterization of "starter" neurons in CRFR1-cre rabies viral tracing experiments. Staining for markers of GPe subtypes reveals "starter" neurons positive for Nkx2.1 (A), PV (B), Npas1 (C), but not FoxP2 (D). Large arrow indicates a "starter" neuron, small arrow indicates a TVA-mCherry expressing neuron that is not infected with rabies virus but expressing molecular marker, Scale bar = 25 μm.

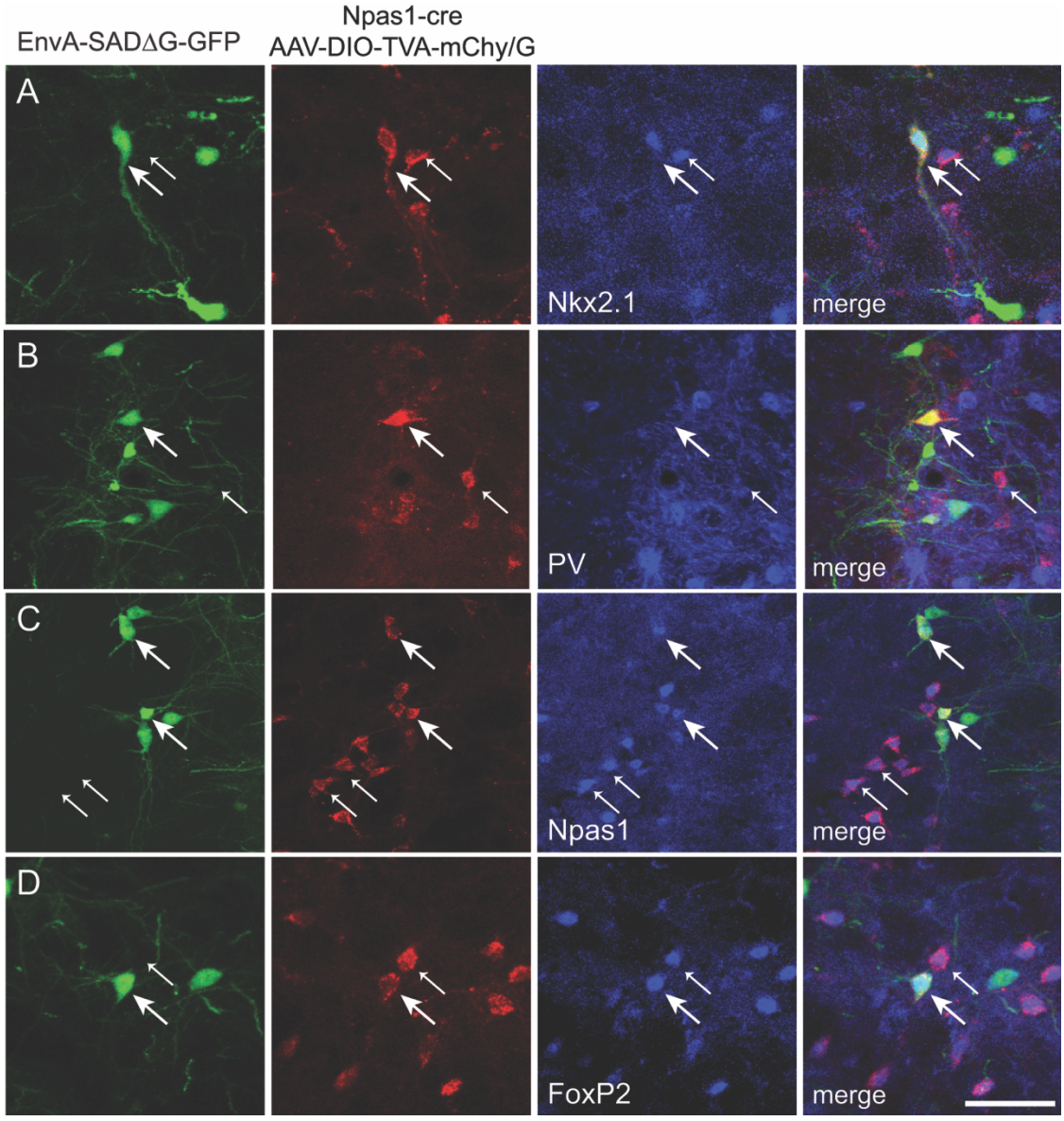

EnvA-SADΔG-GFP
Npas1-cre
AAV-DIO-TVA-mChy/G
A
Nkx2.1
merge
B
PV
merge
C
Npas1
merge
D
FoxP2
merge

Figure 17. Characterization of "starter" neurons in Npas1-cre rabies viral tracing experiments. Staining for markers of GPe subtypes reveals "starter" neurons positive for Nkx2.1 (A), Npas1 (C), and FoxP2 (D). No "starter" neurons were positive for PV (B). Large arrow indicates a "starter" neuron, small arrow indicates a TVA-mCherry expressing neuron that is not infected with rabies virus but expressing molecular marker. Scale bar = 50 μm.

Next, I examined the “traced” neurons in CRFR1-cre mice. I observed that Nkx2.1 (Fig.18 A), PV (Fig.18 B), and Npas1 (Fig.18 C) neurons project to and make synapses on CRFR1 expressing neurons. Noticeably, FoxP2 arkypallidal neurons do not send projections to CRFR1 expressing neurons (Fig.18 D). When I examined “traced” neurons in Npas1-cre mice, I found that Nkx2.1 (Fig.19 A), PV (Fig.19 B), Npas1 (Fig.19 C), and FoxP2 (Fig.19 D) neurons send projections to and make synapses onto Npas1 neurons.

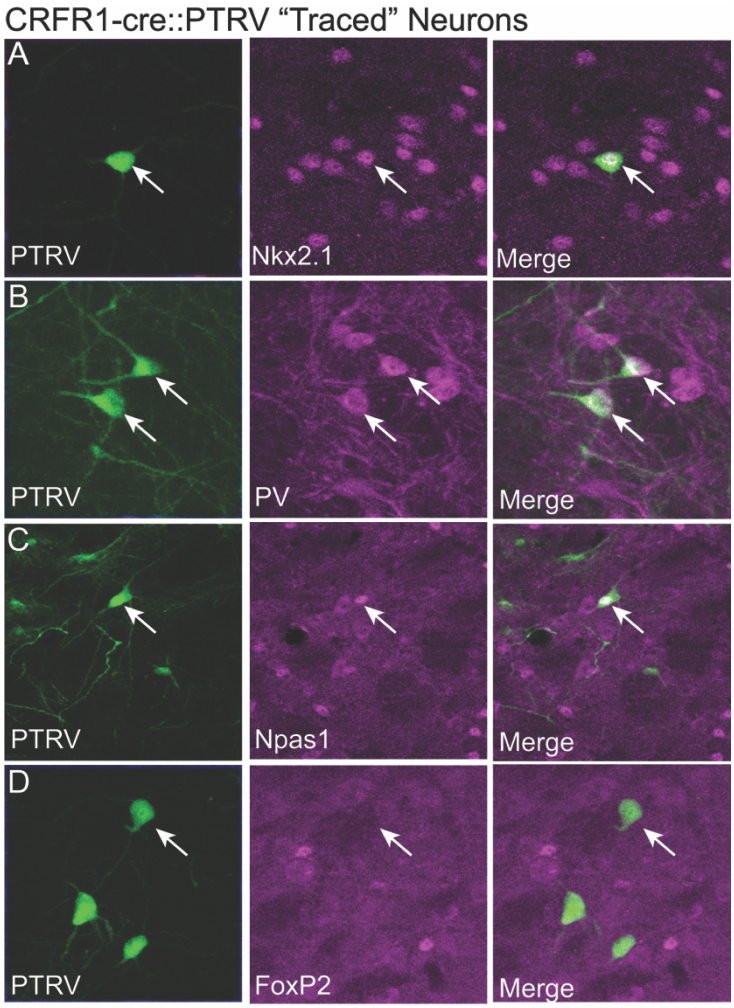


Figure 18. Characterization of “traced” neurons in CRFR1-cre rabies viral tracing experiments. Staining of traced neurons present in the GPe in CRFR1-cre experiments revealed Nkx2.1+ (A), PV+ (B), and Npas1+ (C) “traced” neurons, but not FoxP2+ “traced” neurons (D). Arrows indicate double positive neurons.

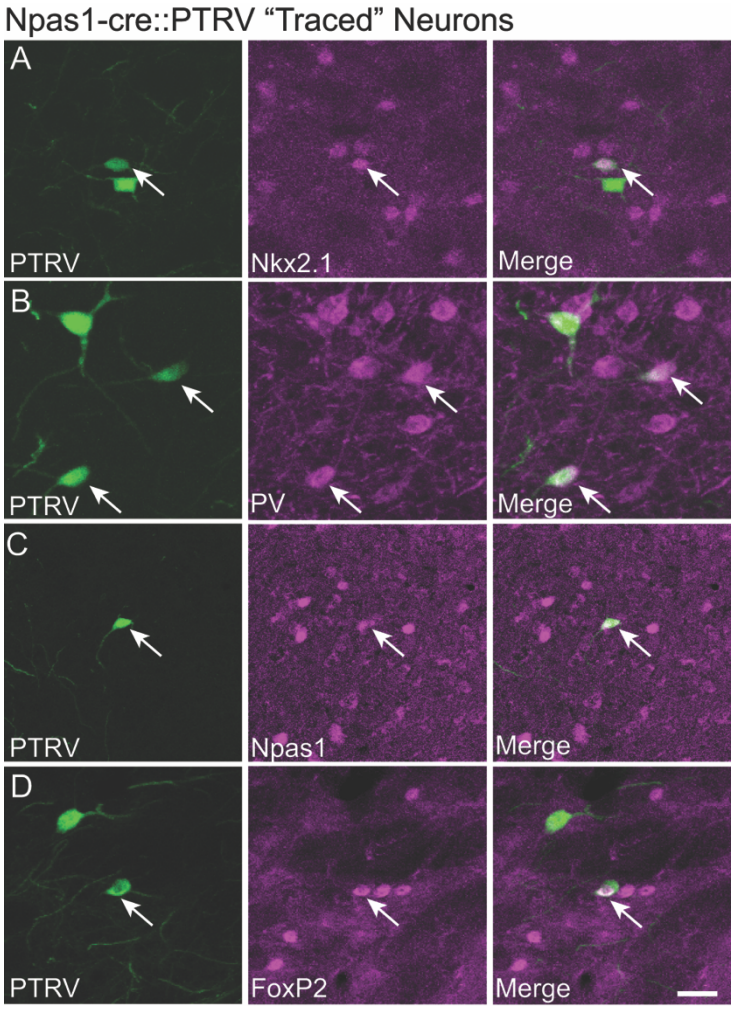


Figure 19. Characterization of "traced" neurons in Npas1-cre rabies viral tracing experiments. Staining of traced neurons present in the GPe in Npas1-cre experiments revealed Nkx2.1+ (A), PV+ (B), and Npas1+ (C) "traced" neurons, as well as FoxP2+ "traced" neurons (D). Arrows indicate double positive neurons. Scale bar = 25 μm for both Fig.18 and Fig.19.

Conclusions

I studied the connectivity of prototypic and arkypallidal neurons of the GPe using PTRV that was specifically expressed in either neuronal cell type. Using rabies mediated viral retrograde tracing, I provide evidence that the second largest source of input to the GPe is the GPe itself.

As the CRFR1-cre line is a novel BAC transgenic in which mCherry expression is a surrogate for Cre expression, it is important to validate that mCherry co-expresses with known markers of CRFR1+ neurons. My evidence shows that Cre expression in the CRFR1-cre transgenic mouse mimics the GFP expression in the CRFR1-GFP transgenic mouse. Specifically, Cre co-expresses with CRFR1+ neuronal markers Nkx2.1, PV, and Npas1. Together, these results demonstrate that CRFR1 expression is restricted to prototypic GPe neurons. In contrast, “starter” neurons in Npas1-cre transgenic mice co-expressed Nkx2.1, Npas1, and FoxP2, but not PV, which is consistent with published reports. In fact, Npas1 and PV have been shown to be distinct non-overlapping classes (Abdi et al., 2015; Dodson et al., 2015; Hernández et al., 2015).

Regarding the connectivity between prototypic and arkypallidal neurons, I demonstrate that CRFR1 expressing prototypic neurons receive projections from prototypic neurons but not arkypallidal neurons. As not all prototypic neurons express CRFR1, it is possible that some prototypic neurons receive projections from FoxP2+ arkypallidal neurons. Although I did not observe any FoxP2+ “traced” neurons that project to CRFR1+ neurons during my extensive visual analysis, it is possible that because CRFR1 is expressed in much greater abundance than FoxP2, I did not

observe any CRFR1+/FoxP2+ neurons that may have been present in my GPe brain sections.

On the other hand, projections from Npas1-cre neurons did not demonstrate selectivity. Specifically, I found that Nkx2.1, PV, Npas1, and FoxP2 neurons project to and make synapses on Npas1 neurons. Previously, I demonstrated that Npas1 neurons can express prototypic or arkypallidal markers. Based on my findings that both subtypes synapse on Npas1 neurons, I hypothesize that these neurons serve to integrate prototypic and arkypallidal processing. For example, a arkypallidal neuron could provide feedback to the STN by synapsing on CRFR1+ prototypic neurons. I also believe that there are likely many uncharacterized neuronal subtypes that contribute to the inter-nuclear circuits of the GPe.

The connectivity within the GPe is likely much more complex than the current state of the field. Future research should include paired electrophysiological recordings between synaptically linked prototypic and/or arkypallidal neurons and the generation of a FoxP2-cre transgenic mouse line. This mouse line could be used to examine whether FoxP2+ arkypallidal neurons synapse on Npas1+ prototypic neurons.

Activation of CRFR1+ Neurons and Npas1+ Neurons has Contrasting Effects on Anxiety-related behavior and Movement

In Brief

Over the course of this study, I have determined that CRFR1 expressing neurons are prototypic GPe neurons. I provided evidence to the novel finding of where CRF input to the GPe originates. I revealed that CRFR1+ and Npas1+ neurons in the GPe receive monosynaptic projections from CRF neurons in the key stress nuclei: the BSTld, CeA, and PVN. Also, I have provided evidence that there is a complex microcircuit within the GPe neuronal subtypes. What remains unanswered is the functional relevance of activation of CRFR1 expressing neurons, which primarily comprise the pallidosubthalamic pathway, and the Npas1 pallidostriatal projections on behavior. Specifically, how do these two GPe neuronal subtypes modulate anxiety-related behavior?

To decipher the functional role of these neuronal subtypes, I took advantage of Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology. This technology allows for the expression of Gq-coupled protein receptors in neurons that exclusively express Cre. These receptors are activated by an inert drug, clozapine N-oxide (CNO). This scheme provides robust activation of neurons expressing Gq-coupled DREADDs. When bound by CNO, Gq DREADDs activate the phospholipase C (PLC) pathway that ultimately results in the release of Ca^{2+} from the endoplasmic reticulum (ER) increasing cytosolic calcium levels leading to activation of neurons. Using the BAC transgenic CRFR1-cre line, I performed experiments to determine the effect on behavior when activating the prototypic population. Using the BAC transgenic Npas1-cre line, I evaluated the functional consequence on behavior of activation of the Npas1+ pallidostriatal/arkypallidal pathway. As I am examining the

stress peptide CRF and its receptor CRFR1, which is responsible for the anxiogenic effect of CRF, I decided to assay my mice in a variety of behavioral paradigms that test for anxiety-related behavior as well as movement. I infected CRFR1-cre and Npas1-cre mice with Cre-dependent DREADD-Gq virus (AAV-EF1a-DIO-hM3Dq-mCherry) in the GPe (Armbruster et al., 2007). This allowed for the expression of Gq DREADDs in either CRFR1 neurons or Npas1 neurons depending on the mouse strain.

Results

In CRFR1-cre mice, the viral infection led to GPe-specific expression of hM3Dq-mCherry in Nkx2.1+ and PV+ prototypic neurons (Fig.20 A, B). In Npas1-cre, the majority of GPe neurons expressing DREADD-Gq were Npas1+/FoxP2+ (Fig.20 C, D).

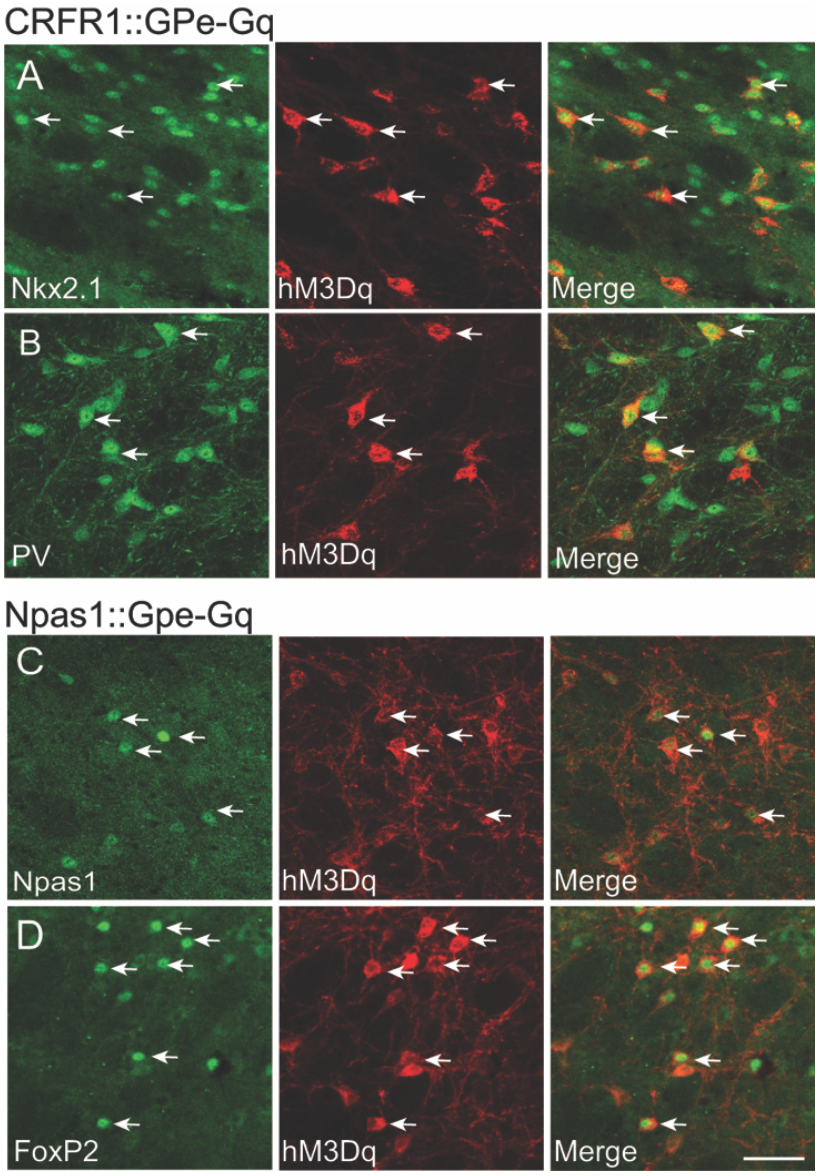


Figure 20. Confirmation of neuronal DREADD Expression.

In CRFR1::GPe-Gq mice, I verified that prototypic neurons were expressing DREADDs using markers Nkx2.1 (A) and PV (B). In Npas1::GPe-Gq mice, I verified expression by Npas1+ neurons (C) and arkypallidal neurons labeled by FoxP2 (D). Scale bar = 25 μm

After confirming the expression of prototypic neurons in CRFR1::GPe-Gq mice and Npas1+/FoxP2+ arkypallidal neurons in Npas1::GPe-Gq mice, I collected data from a battery of behavioral paradigms testing for anxiety-related behavior and in some cases movement as well. Before performing any behavioral test, I allowed the mice to acclimate to the testing room and then administered an intraperitoneal (IP) injection of CNO. I waited fifteen minutes for the CNO to robustly activate the DREADD-Gq expressing GPe neurons before the commencement of testing.

I first tested the mice in the Light Dark Transfer Assay (LDT). This test consists of an enclosed arena with a light side and a dark side separated by a hole large enough for the mouse to easily pass. The LDT measures anxiety-related behavior by the mouse's level of aversion to the light side of the maze. Specifically, the LDT measures passive avoidance of stressful situations. As mice are nocturnal, they prefer the dark side of the arena versus the light side. The more times a mouse transfers from the dark side to the light side of the maze, a decrease in anxiety-related behavior is inferred (Cryan and Holmes, 2005).

I found an opposing effect on anxiety-related behavior when CRFR1::GPe-Gq neurons or Npas1::GPe-Gq neurons were activated. In CRFR1::GPe-Gq mice, I observed a significant decrease in the number of light entries compared to CRFR1-cre control virus injected mice (Cre-dependent mCherry) (10.83 ± 1.48 vs. 19.11 ± 2.29 .88, $t_{(19)}$=2.81, $p<0.05$), suggesting activation of CRFR1+ GPe neurons elevates anxiety-related behavior (Fig.21). Of note, I also tested CRFR1::GPe-Gq and Npas1::GPe-Gq mice injected with saline and found no difference between control virus mice injected with CNO and DREADD-Gq mice

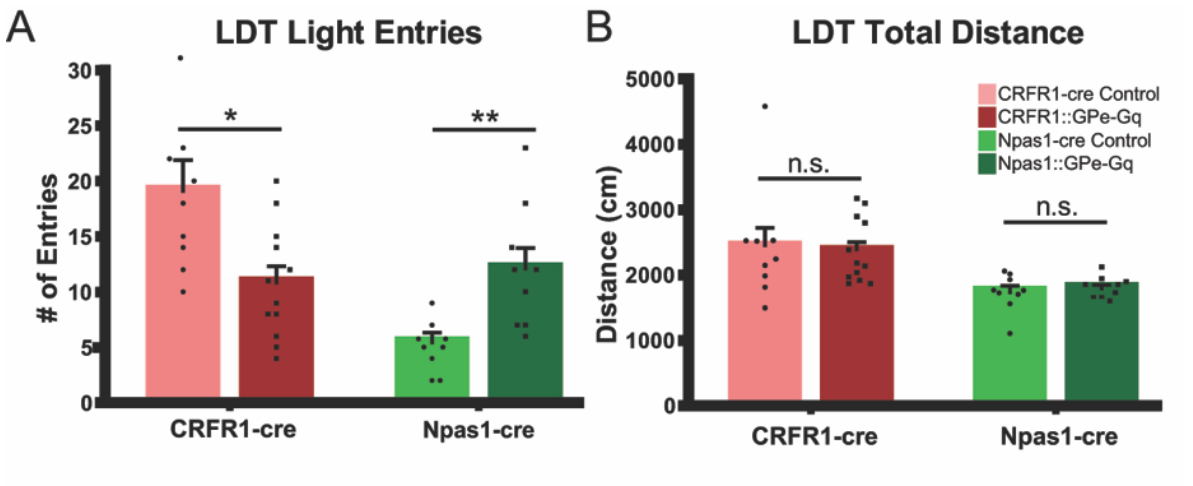


Figure 21. Activation of CRFR1 and Npas1 neurons has opposing effects on anxiety-related behavior on the LDT. (A) On the LDT, I saw a decrease in light entries in CRFR1::GPe-Gq mice (dark red) and an increase in Npas1::GPe-Gq mice (dark green), indicating an opposing effect of activation of CRFR1 or Npas1 GPe neurons on anxiety-related behavior. (B) I observed no difference between experimental and control animals in total distance traveled for either transgenic mouse line. CRFR1-cre: CRFR1::GPe-Gq n=9, n=12 control. Npas1-cre: Npas1::GPe-Gq n=9, n=9 control.

injected with saline, therefore the controls were combined for all experiments. In contrast, I observed a significant increase in the number of light entries in Npas1::GPe-Gq mice compared to control Npas1-cre control virus injected mice (12.11 ± 1.87 vs. 5.44 ± 0.87, $t_{(16)}$=3.24, p<0.01), indicating that Npas1+ neuron activation decreases anxiety-related behavior (Fig.21 A). In both CRFR1::GPe-Gq mice and Npas1::GPe-Gq mice, I did not observe a difference in total distance traveled on the LDT (Fig.21 B).

To assess active expressions of anxiety-related behavior, I used the marble burying task in which anxiety is measured as a function of the number of marbles buried in bedding. The more marbles buried, it is inferred that the mouse is displaying greater anxiety-related behavior (Kedia and Chattarji, 2014). With activation of CRFR1::GPe-Gq neurons, the number of marbles buried was significantly increased compared to control infected mice (82.50 ± 6.02 % vs. 38.57 ± 4.04 %, $t_{(15)}$=5.50, p<0.001) (Fig.22 A). With activation of Npas1::GPe-Gq neurons, the number of marbles buried was significantly decreased compared to corresponding Npas1-cre control mice (40.00 ± 4.08 % vs. 76.00 ± 8.12 %, $t_{(7)}$=3.64, p<0.01) (Fig.22 A).

In both the LDT and marble burying task, I consistently observed a baseline anxiety phenotype in Npas1-cre mice making direct comparisons between the two Cre lines not possible. It is possible that the difference in baseline anxiety is a result of the location of the gene insertion when the BAC transgene was generated. Also, it is plausible that Cre expression in neurons may alter their baseline function. By backcrossing Npas1-cre mice to C57BL/6 mice, I may be able to assess if the resulting phenotype is an artifact of a mixed background created from generations of

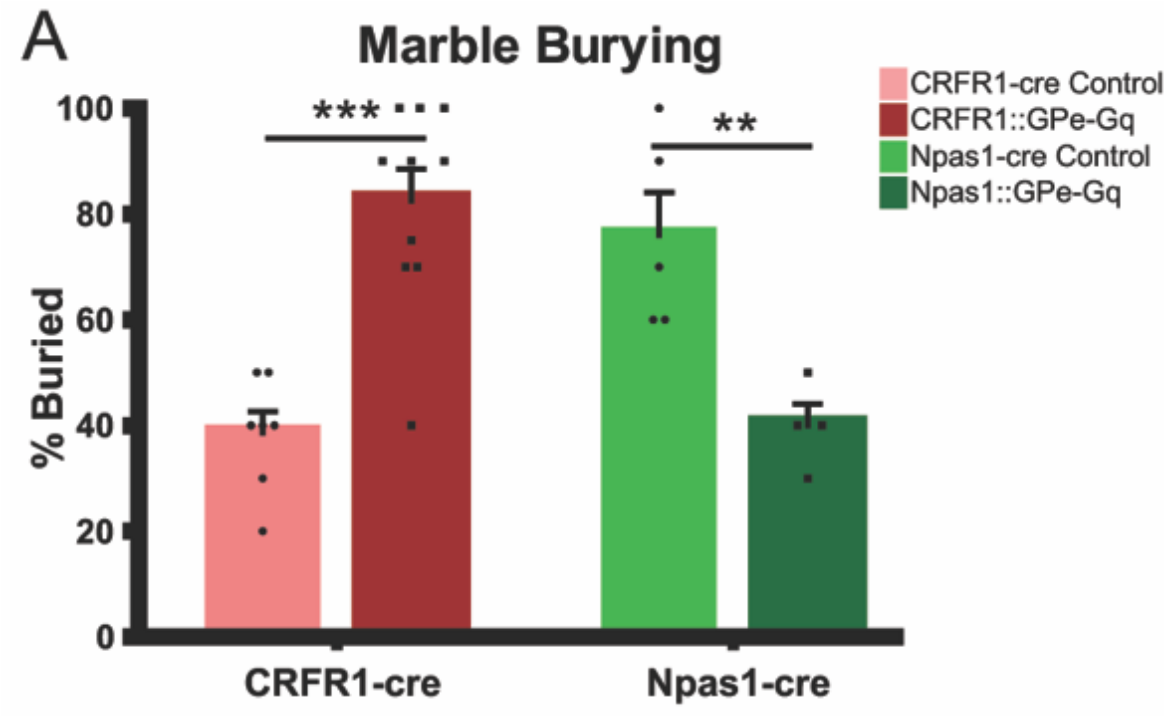


Figure 22. CRFR1 and Npas1 neuron activation have opposing effects on marble burying. (A) Activation of CRFR1::GPe-Gq neurons resulted in significantly more marbles buried than CRFR1-cre controls, while Npas1::GPe-Gq mice buried significantly less marbles than their corresponding Npas1-cre controls. Note the difference between the two unmanipulated Cre lines. CRFR1-cre: CRFR1::GPe-Gq n=7, n=10 control. Npas1-cre: Npas1::GPe-Gq n=5, n=4 control.

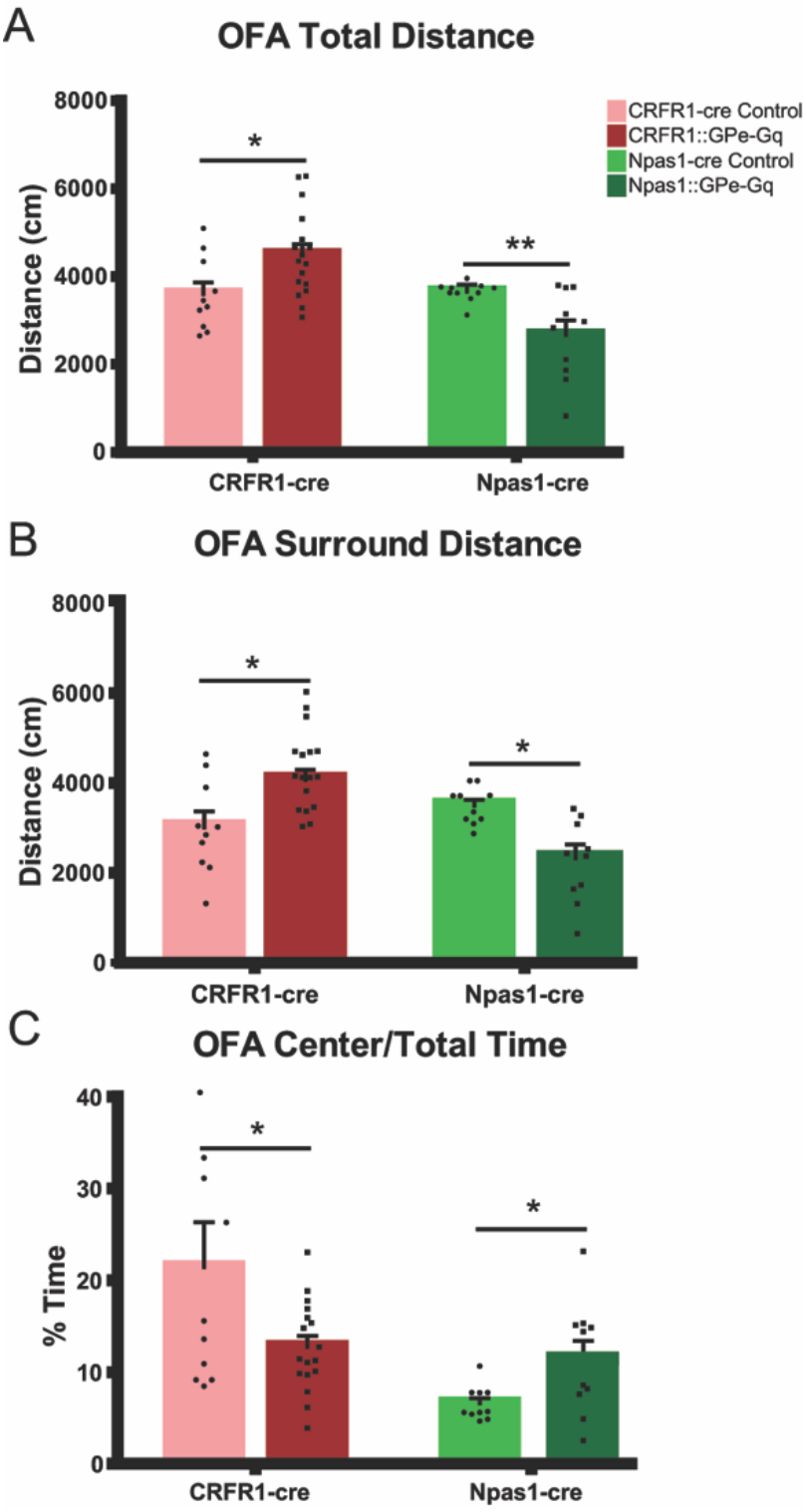
breeding. However, both tests support the conclusion that activating CRFR1+ GPe neurons elevates anxiety-related behavior while activating Npas1+ GPe neurons decreases anxiety-related behavior.

While I did not see changes in the total distance traveled on the LDT between control and CNO-treated mice (Fig.21 B), I consistently observed more movement in CRFR1::GPE-Gq mice and less movement in Npas1::GPe-Gq mice. To further explore how activation of these neuronal populations impact activity, I measured movement in the relatively low anxiety eliciting context of the open field assay (OFA) (Bale et al., 2000). The open field consists of an empty arena with plexi glass siding. The mouse can stay close within the walls of the maze (protected due to thigmotaxis), which represents a greater level of anxiety-related behavior, or venture into the open center region (unprotected) representing decreased anxiety-related behavior.

With activation of CRFR1 neurons, I observed a significant increase in the total distance traveled in CRFR1::GPe-Gq mice (4498.94 ± 236.00 cm vs. 3602.70 ± 265.36 cm, $t_{(25)}$=2.43, $p<0.05$) (Fig.23 A). Conversely, with activation of Npas1+ neurons, I observed a significant decrease in total distance traveled in Npas1::GPe-Gq mice (2675.00 ± 322.66 cm vs. 3652.60 ± 69.85 cm, $t_{(18)}$=2.96, $p<0.01$) (Fig.23 A). However, comparing CRFR1::GPe-Gq mice to Npas1::GPe-Gq mice, movement was biased toward protected or exposed regions of the open field, suggesting a strong anxiety component to the movement. Specifically, in CRFR1::GPe-Gq mice, I observed elevated levels of movement in the surround regions (protected) of the open field (4065.41 ± 229.49 cm vs. 3012.00 ± 351.90 cm, $t_{(25)}$=2.62, $p<0.05$) (Fig.23 B) and a lower center/total time ratio (12.7 ± 1.2 % vs. 21.4 ± 4.9 %, $t_{(25)}$=2.154, $p<0.05$)

(Fig.23 C). The higher the center/total time ratio, it is interpreted to represent decreased anxiety-related behavior (Bale et al., 2000). In Npas1::GPe-Gq mice, I saw the opposite effect; mice moved less in the surround region of the maze (2323.60 ± 304.45 cm vs. 3489.50 ± 2.29 cm, $t_{(18)}$=3.51, p<0.01) (Fig.23 B) and displayed a higher center/total time ratio (11.5 ± 1.9 % vs. 6.6 ± 0.6 %, $t_{(18)}$=2.39, p<0.05) (Fig.23 C).

In summary, activation of CRFR1 neurons in the GPe biases the mice toward increased movement in protected regions of the maze, while activation of Npas1 GPe neurons causes animals to decrease their movement and bias towards open, less protected areas of the maze (Fig.24 A). These data demonstrate that activation of CRFR1 neurons increases anxiety-related behavior and movement while Npas1 neuronal activation leads to a decrease in anxiety-related behavior and movement.


A
OFA Total Distance
CRFR1-cre Control
CRFR1::GPe-Gq
Npas1-cre Control
Npas1::GPe-Gq
Distance (cm)
8000
6000
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2000
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CRFR1-cre
Npas1-cre
B
OFA Surround Distance
Distance (cm)
8000
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2000
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Npas1-cre
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OFA Center/Total Time
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40
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0
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*
CRFR1-cre
Npas1-cre

Figure 23. CRFR1 and Npas1 neuron activation has opposing effects on anxiety-related behavior in the open field assay. (A) CRFR1::GPe-Gq animals displayed more total movement in the open field than controls, while Npas1::GPe-Gq animals displayed less movement than controls. (B) This movement was biased toward particular regions of the maze, as CRFR1::GPe-Gq animals moved more in the surround regions of the maze and Npas1::GPe-Gq mice moved less in the surround. (C) This difference resulted in a decrease in the center time ratio in CRFR1::GPe-Gq mice and an increase in center time ratio in Npas1::GPe-Gq mice, indicating elevated and decreased anxiety-related behavior, respectively. CRFR1-cre: CRFR1::GPe-Gq n=10, n=17 controls, Npas1-cre: Npas1::GPe-Gq n=10, n=10 controls.

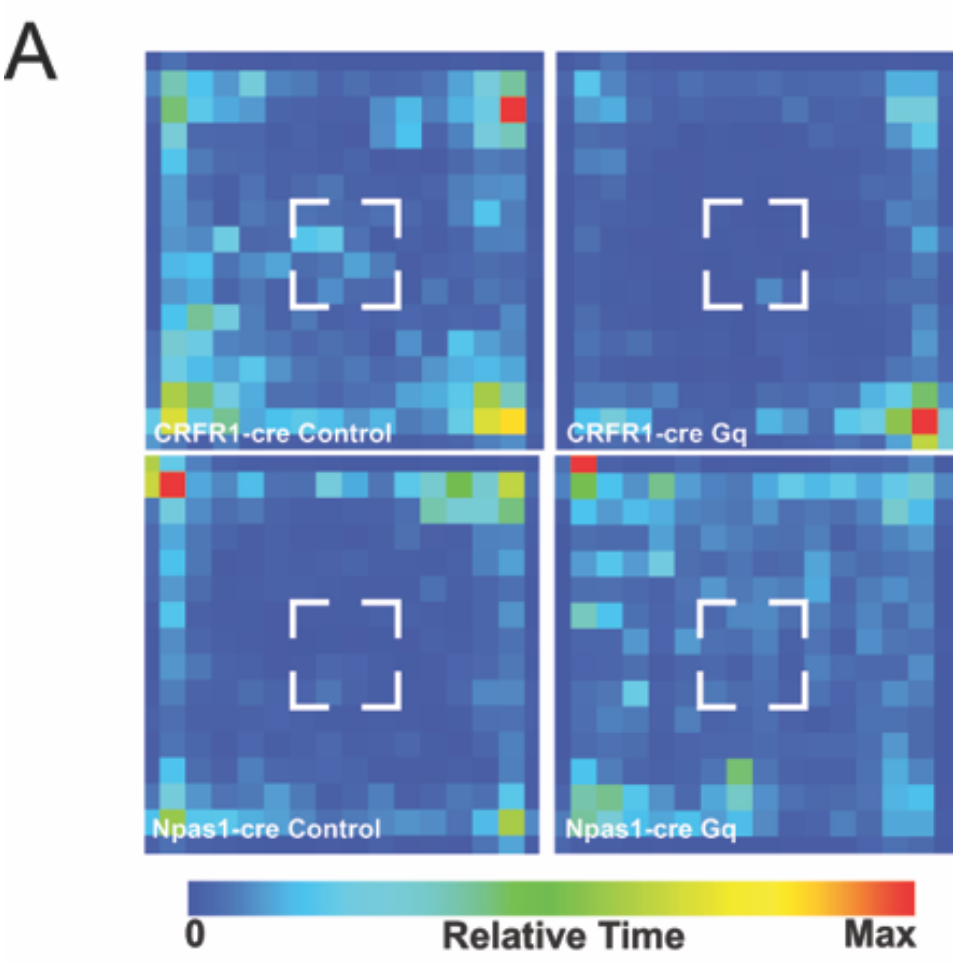

Figure 24. Heatmaps of time duration at locations on OFA. (A) Heatmaps of example open field assays display biasing of movement to opposite regions of the maze with activation of CRFR1 GPe neurons (top maps), and Npas1 GPe neurons (bottom maps). Less time is indicated by blue and more time is indicated by red. CRFR1::GPe-Gq mice spend less time in the center compared to controls suggesting an increase in anxiety-related behavior. Npas1::GPe-Gq mice spend more time in the center compared to controls interpreted as a decrease in anxiety-related behavior.

Conclusions

We tested CRFR1::GPe-Gq and Npas1::GPe-Gq mice on a battery of behavioral paradigms used to measure anxiety-related behavior and mobility including the LDT, OFA and marble burying task. If I had tested the mice in a purely motor task such as the DigiGait, which measures the speed and pace of movement, I may have never observed the anxiety phenotype (Berryman et al., 2009). All three task indicated that activation of CRFR1 neurons increases anxiety-related behavior and activation of Npas1 neurons decreases anxiety-related behavior. Specifically, in the LDT assay, mice with activated CRFR1 neurons explore the illuminated section less than mice with activated Npas1 neurons. While the LDT assay measures passive anxiety (baseline anxiety), the marble burying task is an indicator of active anxiety because the animal actively relieves themselves of the stress producing, noxious agent i.e. by burying the marbles. In the marble burying task, I observed that activation of CRFR1 neurons significantly increases active anxiety-related behavior, while Npas1 neuron activation reduces active anxiety-related behavior. Finally, the OFA was very informative about the animal's anxiety level. CRFR1 neuron activation leads to a hypermobile, increased anxiety-related behavior state. Specifically, the hypermobility occurred in the more protected region of the maze. In contrast, Npas1::GPe-Gq animals move less in the surround protected region of the maze and venture into the open field more often indicating lower anxiety-related behavior.

I did not observe a difference in total distance traveled in CRFR1::GPe-Gq and Npas1::GPe-Gq mice on the LDT, while I did observe this phenomenon on the OFA. A possible reason for this result is that the LDT is not a test of general mobility. Its

primary use is to determine a mouse's relative anxiety-related behavior by the number of transitions into the light. The interpretation that the mice are more anxious is based on their lack of transitions into the light region; Additionally, the way the LDT is recorded and scored may obscure the movement component of the anxiety-related behavior.

On the OFA, there is no region for the mice to "hide" such as on the LDT. The increase in distance traveled while the mice are displaying thigmotaxic behavior in the case of CRFR1::GPe-Gq compared to control mice may represent an active avoidance of the center anxiety eliciting region. The interpretation that CRFR1::GPe-Gq mice are displaying an increase in movement in the OFA as a component of anxiety is strengthened by my results on the marble burying task, which is a measure of active anxiety. It is likely that I would have observed increased movement in the CRFR1::GPe-Gq mice as more movement is required to bury more marbles.

I interpreted the increased movement in the OFA surround region by the CRFR1::GPe-Gq mice as increased anxiety-related behavior based on contrasting behavior manifested due to activation of CRFR1 or Npas1 neurons. Specifically, anxiety manifests as increased movement on the OFA in CRFR1::GPe-Gq mice and the decreased movement in Npas1::GPe-Gq mice. More behavioral paradigms such as the elevated-plus maze (EPM), which measures anxiety-related behavior by the mice's time in an open, exposed region of the maze elevated off the floor, may help resolve this issue. Due to experimental limitations, I was unable to perform this paradigm.

The PVN is not known to play a direct role in the behavioral outcomes of the initiation of the stress response. The PVN is the activator of the endocrine (HPA-axis) and autonomic cascade that is the stress response. The possibility that it plays a direct role in the experience of anxiety is novel (Dabrowska et al. 2011). Interestingly, the PVN may release fast acting excitatory glutamate with CRF at the initiation of the stress response to increase both anxiety-related behavior and movement. As I have demonstrated, CRF neurons in the PVN preferentially target CRFR1+ prototypic neurons while CeA CRF neurons preferentially target Npas1+/arkypallidal neurons (Fig.14 A). Intriguingly, overexpression of CRF in the CeA leads to a reduction in stress-induced anxiety (Kormos and Gaszner 2013). The CeA may release CRF into the GPe to activate the “Stop” function of the arkypallidal pathway when a stress response is no longer beneficial or the stressor is insufficiently noxious (Mallet et al., 2016). While the activation of the stress response promotes survival, the modification of bodily functions induced by the stress response are only beneficial for a limited period of time. As CRFR2 (which may oppose the actions of CRFR1) is not expressed in the GPe, the only way to terminate the GPe-mediated stress response may be through classic BG function of inhibiting unwanted actions (Kormos and Gaszner, 2013). Activation of the Npas1+/arkypallidal pathway may serve to stop the stress response.

As I used Gq DREADDs to activate CRFR1 or Npas1 neurons, further study is needed to provide evidence that it is in fact CRF that is released from the PVN or the CeA into the GPe that mediates the anxiogenic or anxiolytic effects. One means of addressing this question is to artificially stimulate CRF neurons in either the PVN or

CeA using Cre-dependent Channelrhodopsin (ChR2). ChR2 is a non-specific cation channel that opens in response to blue light delivered by a fiber optic implant (Boyden et al., 2005). To perform this experiment, ChR2 would be expressed in CRF PVN neurons in CRF-cre mice introduced by bilateral stereotaxic injection. The fiber optic would be placed in the GPe and upon blue light stimulation, I anticipate CRF will be released and I would assess for anxiety-related behavior. To ensure that it is actually CRF and not a fast acting neurotransmitter mediating the behavior, a CRFR1 specific antagonist such as R121919 would be administered to the mouse for several days to allow the antagonist to reach steady state prior to testing (Gutman, 2003). Similar experiments could be performed with ChR2 in CRF neurons in the CeA. These experiments will allow a more detailed assessment of functional consequences of activation of distinct CRF neuronal subtypes. While DREADDs have a long lasting effect on neuronal activity, ChR2 has control of neuronal activation at the millisecond time scale (Armbruster et al., 2007; Boyden et al., 2005). Using both techniques together would strengthen any conclusion drawn by a single technique.

In closing, I have provided evidence of a novel function of the GPe in its ability to confer bidirectional control over anxiety-related behavior through CRFR1 and Npas1 neurons.

Discussion

Experimental Conclusions

Throughout the course of this study, I have made many novel discoveries. First, I performed electrophysiological experiments to verify that only GFP fluorescent neurons in the CRFR1-GFP mouse respond to CRF application with a robust increase in firing frequency. Using a specific CRFR1 antagonist, I confirm this receptor mediates the increase in firing rate of GFP labeled CRFR1 expressing neurons. Second, using the BAC transgenic mouse line CRFR1-GFP, I provide evidence that CRFR1 expressing GPe neurons are of the prototypic subtype. I provide evidence that CRF-positive neurons send projections that terminate in close proximity to CRFR1 expressing neurons in the GPe. To validate that synapses are formed between CRF neurons and CRFR1+ neurons, I performed rabies viral mediated retrograde tracing using the novel BAC transgenic CRFR1-cre mouse line. I provide evidence that the source of this CRF input to the GPe is three major stress regions: BST, CeA, and PVN. As I observed a small population of Npas1 neurons express CRFR1, I performed the identical experiment in the BAC transgenic mouse line Npas1-cre. I found that Npas1+ neurons receive projections from the same stress regions as CRFR1+ neurons. As CRFR1+ prototypic neurons and Npas1 neurons project to different brain regions, I hypothesized that the CRF projections onto different neuronal subtypes serve different functions and may have different outcomes on behavior. Consistent with this hypothesis, I provide evidence that CRFR1+ and Npas1+ neurons receive differential input from the major stress nuclei. More connections from the PVN are made onto CRFR1+ prototypic neurons while more connections from the CeA are made onto Npas1+ neurons. A limitation to this experiment is that I cannot say that all

of the projecting neurons are CRF neurons. This limitation is due to the fact that CRF neuron staining is complicated by the fact that CRF is a peptide located throughout the axon which makes cellular staining difficult. I tried to mitigate this limitation by ICV colchicine injection to stop vesicular transport allowing CRF to accumulate in the cell body, but as a result, I am only able able to visualize some CRF neurons.

I observed that the GPe contains a microcircuit where CRFR1 prototypic neurons receive projections only from prototypic neurons and do not receive projections from arkypallidal neurons. The microcircuit formed by Npas1 neurons is less clear, but I speculate that they may form a bridge between prototypic and arkypallidal neurons or possibly that arkypallidal neurons only send projections and synapse on other arkypallidal neurons, in addition to their STR target. This would create a minimum of two circuits in the GPe in support of my hypothesis that the two neuronal subtypes serve different functions. Finally, I performed behavioral testing of both Cre lines in anxiety-related paradigms. I activated CRFR1+ neurons and found that anxiety-related behavior and movement in protected regions of the arena are increased. I activated Npas1+ neurons and found the opposite behavior. Npas1+ neuron activation led to reduced anxiety-related behavior and movement. Combining the conclusions from all three behavioral paradigms (LDT, marble burying, and OFA), I uncovered that the GPe likely contributes to the stress response and mediates anxiety-related behavior through different neuronal subtypes. This study has provided novel findings to both the BG and stress research fields and will serve as the basis for future study into the GPe-mediated stress response and anxiety-related behavior. In

the next sections, we will explore some of these ideas and possibilities that may arise when these circuits are dysfunctional.

Novel Circuits

I have provided novel evidence that three of the major stress nuclei (BSTld, PVN, and CeA) are sources of CRF neuronal input to the GPe. Moreover, different GPe neuronal subtypes receive differential synaptic input from these major stress nuclei (Fig.25 A). Npas1 expressing neurons receive a greater proportion of CRF input from the CeA while CRFR1 expressing neurons receive a greater proportion of CRF input from the PVN. The question is raised of how the stress response is affected by this novel circuitry. It is plausible that at the initiation of the stress response PVN neurons releasing CRF into the hypophyseal portal may also release CRF into the GPe. This release may happen either through volume diffusion or synaptically, or possible by both mechanisms. Activation of these neurons by CRF may serve to increase both anxiety-related behavior and movement effectively alerting an organism to a potential threat and biasing BG output to allow the anxiety component of movement to dominate motor programs. To restore homeostasis, CRF released from CeA neurons may act on the small population of CRFR1+ Npas1 neurons to initiate an anxiolytic cascade propagated by arkypallidal GPe neurons. Alternatively, CRF activation of Npas 1 neurons may be delayed due to CRF from the PVN diffusion time through the GPe. The initial wave of CRF release would activate the anxiogenic circuit of the GPe because CRFR1 is more abundantly expressed in prototypic neurons than Npas1 neurons. However, with time anxiolytic Npas1 neurons would eventually activate effectively reducing the anxiety initiated from the first burst of CRF from the PVN as it has time to diffuse and excite the small population of Npas1 neurons.

I also provide evidence that a complex microcircuit exists within the GPe (Fig.25 B). My data illustrates that CRFR1 expressing neurons only receive input from prototypic neurons and not from arkypallidal neurons. In contrast, Npas1 neurons receive input from both prototypic and arkypallidal neurons raising the intriguing hypothesis that Npas1 neurons may serve as a link between prototypic and arkypallidal neurons.

As the GPe is GABAergic, it is anticipated that all internuclear communication between neurons would be inhibitory. However, our current models of the BG do not take into account different neuronal subtypes. In fact, I observed an increase in movement as measured by the OFA following activation of CRFR1 prototypic neurons and a decrease in movement following activation of Npas1 neurons. In the classic model of the BG, increased activation of the pallidosubthalamic-projecting neurons as caused by a decrease in D2 inhibition on the GPe should decrease movement, but I hypothesize that the observed increase in movement is purely a manifestation of anxiety. This movement process may be mediated by a limbic component of the GPe that may intersect with motor circuits of the BG.

My findings require that we rethink the classical BG model of D1 direct and D2 indirect pathways. For many years, researches used this model to explain the movement phenotypes manifested in patients with neurodegenerative disorders like PD. However, with advances in technology, we now can dissect the neural circuitry of the BG to better understand how it mediates movement and other components of behavior. My neural tracing and behavioral data suggest that the BG has greater complexity than the two pathway model. While the D1/D2 pathway model has been

useful, it has become antiquated. Our job as researchers is to modify and add to existing concepts and models using new techniques and tools. My data supports a new model of the BG in which GPe neuronal subtypes may display differential responses to the CRF stress peptide to elicit contrasting behavioral and movement outcomes.

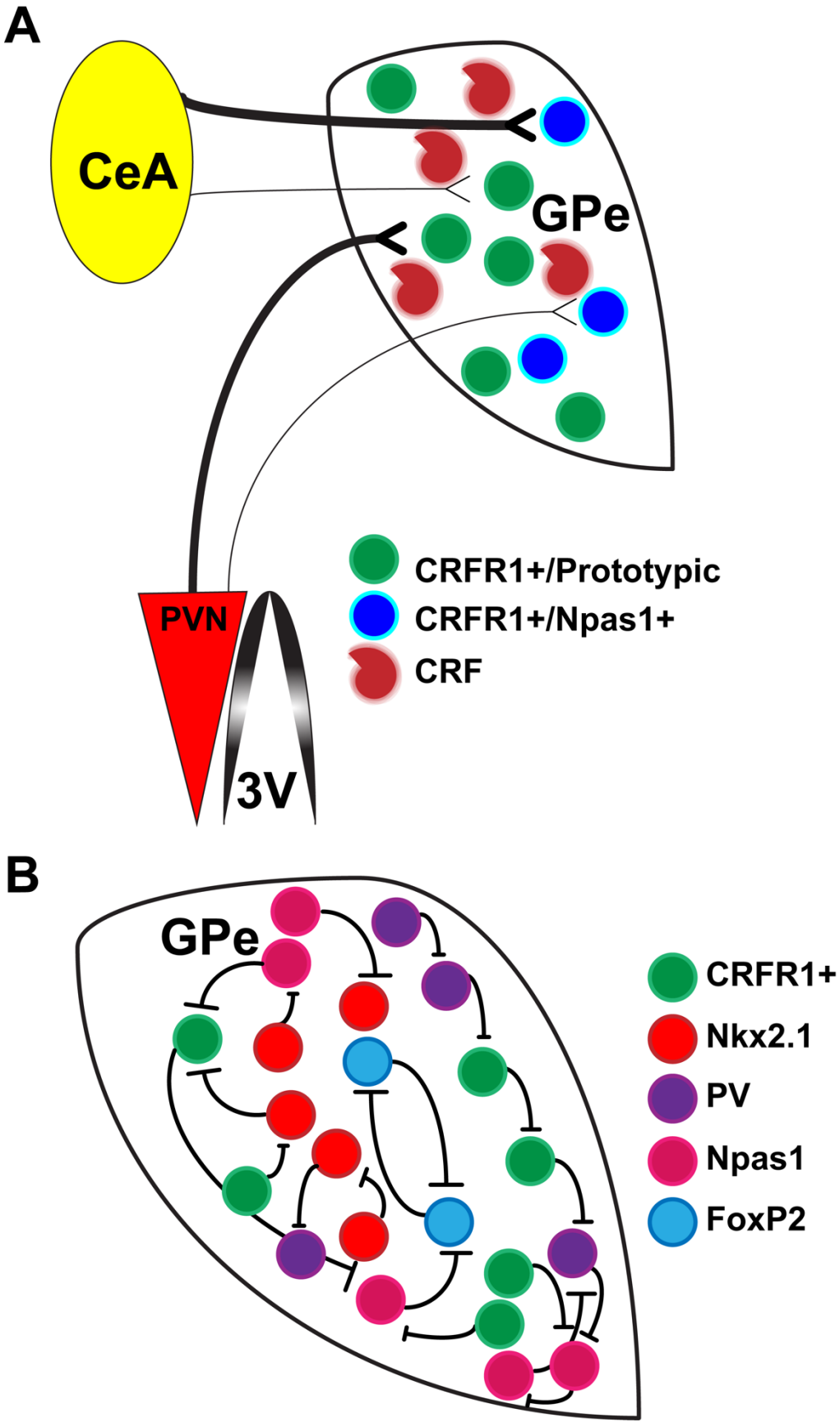
A
CeA
GPe
PVN
3V
CRFR1+/Prototypic
CRFR1+/Npas1+
CRF
B
GPe
CRFR1+
Nkx2.1
PV
Npas1
FoxP2

Figure 25. Novel Circuits. (A) The PVN sends more projections to CRFR1 expressing neurons in the GPe than the CeA while the CeA sends more projections to Npas1 expressing neurons possibly allowing disparate functions during the stress response. (B) CRFR1 expressing neurons and prototypic neurons receive projections and send projections only to other prototypic neurons. FoxP2 arkypallidal neurons project to other FoxP2 arkypallidal neurons. Npas1 neurons act as a bridge allowing prototypic information to reach FoxP2 arkypallidal neurons. GPe = external globus pallidus; CeA = central amygdala; PVN = paraventricular nucleus of the hypothalamus; 3V = 3rd ventricle.

Dysregulation of CRF and the HPA-axis Leads to Neuropsychiatric Disease

Psychiatric illnesses such as anxiety disorders and depression are among the most common mental disorders and present a serious public health epidemic (Bonfiglio et al., 2011; Risbrough and Stein, 2006). Those who suffer from anxiety disorders often are severely disabled by the disease and experience a profound reduction in quality of life. Stress is an etiologic factor in psychiatric illnesses, especially in anxiety disorders. Therefore, the study of stress, anxiety-related behavior, and the stress response is crucial to further our understanding of the mechanisms underlying psychiatric illnesses (Aubry, 2013; Binder and Nemeroff, 2009). Many anxiety-related disorders are believed to originate from dysfunction of neural circuits that respond to stress (Ressler and Mayberg, 2007). The circuits drive stress hormone secretion via the HPA-axis (Anthony et al., 2014; Risbrough and Stein, 2006; Tovote et al., 2015). They also project directly to important stress-activated nuclei such as the amygdala and BST to directly modulate behavior (Flandreau et al., 2012; Stokes and Sikes, 1991). As discussed, the stress-responsive circuitry is unified by the expression and localization of CRF and its receptors CRFR1 and CRFR2.

Dysregulated CRF circuits are involved in neuroendocrinological disturbances and over 20 years of preclinical studies have pointed to CRF as a major factor in psychiatric illnesses including anxiety and depression (Binder and Nemeroff, 2009; Risbrough and Stein, 2006). CRF expression has been shown to be elevated in depressed individuals. Also, elevated levels of CRF have been detected in the cerebrospinal fluid (CSF) of depressed individuals, which is due to overactive extra-hypothalamic CRF neurons (Arborelius, 1999). These finding have spurred the

investigation of antagonism of the receptor responsible for the anxiogenic effects of CRF, CRFR1 (Hauger et al., 2009). CRFR1 antagonism has not been successful in clinical trials pointing to the fact that we still do not understand the complexity of CRF action and how CRF acts to drive anxiety, anxiety-related disease, and other psychiatric illnesses (Howerton et al., 2014).

When CRF is hypersecreted, the originally beneficial effects of HPA-axis activation turn into increased risk for the development of depression- and anxiety-like behavior (Landgraf, 2006). In transgenic mice that overexpress CRF, there are noticeable depression- and anxiety-related symptoms (Britton et al., 1986; Pepin et al., 1992; Ströhle et al., 1998). In addition, CRF overexpression has been linked to anxiety and hyperlocomotion (Coste et al., 2001; Stenzel-Poore et al., 1996). Interestingly, I observed the same behavior following DREADD activation of CRFR1 neurons in the GPe. It is possible that DREADD mediated chronic activation of CRFR1 in the GPe mimics the effects of CRF oversecretion.

Excessive CRF secretion leads to HPA-axis overactivity (increased release of ACTH and Cort) which occurs in 50-80% of individuals diagnosed with depression and therefore the functioning of the HPA-axis has received attention as a potential biomarker for depression (Heuser et al., 1994). Moreover, activation of the HPA-axis activity may be a useful indicator of treatment response because HPA-axis hyperactivity is correlated to an increased risk for a reoccurrence of depression (Zobel et al., 2001). Hypersecretion of CRF leads to increased Cort, therefore glucocorticoids have been linked to depression. Although higher Cort is most often seen in patients with depression, CRF dysregulation can lead to greater or diminished Cort secretion

(Abelson et al., 2006; Cookson et al., 1985; Vincent and Jacobson, 2014). HPA-axis dysregulation that leads to increased ACTH secretion has also been observed in individuals with panic disorder, an extreme form of anxiety disorder in which patients experience disabling panic attacks sometimes without environmental cues or an apparent stressor (Abelson et al., 2006).

As the prefrontal cortex (PFC) plays a primary role in translating stressful emotional information into action, dysregulation of GRs in the PFC has been implicated in major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) (McKlveen et al., 2013). Additionally, the PFC, which interprets emotional stress levels, expresses abundant GRs which are proposed to be regulators of feedback control of the HPA-axis (Diorio et al., 1993; Fuxe et al., 1985; Meaney et al., 1985; Radley et al., 2006; Reul and de Kloet, 1986). This indicates that overactivity of GRs in the PFC may play a role in pathological negative feedback to the HPA-axis. In addition, chronic activation of PFC GRs by chronic stress disrupts GR expression resulting in dendritic atrophy, spine loss, and altered neuronal excitability, which causes a loss of PFC HPA-axis regulation (Goldwater et al., 2009; Mizoguchi et al., 2003; Radley et al., 2008, 2004). This suggests that elevated levels of Cort secretion in the PFC may play a role in stress-related diseases such as depression and anxiety (Pariante and Miller, 2001; Price and Drevets, 2012).

To test the negative feedback of the HPA-axis, the dexamethasone suppression test (DST) uses the synthetic glucocorticoid dexamethasone. In depressed patients, the activity of the drug does not suppress levels of ACTH and Cort providing a possible mechanism by which the HPA-axis is hyperactive. In

contrast, DST suppresses levels of ACTH and Cort in control individuals indicating an intact negative feedback in the HPA-axis. In a mouse model of high anxiety-related behavior and depressive behavior, it was found that the mice experienced a lower level of HPA-axis reactivity (Arborelius, 1999; Sotnikov et al., 2014). In sum, any dysregulation in the HPA-axis can lead to behaviors experienced by individuals with psychiatric diseases (Arborelius, 1999).

A more sensitive neuroendocrine test to reveal HPA-axis dysregulation combines the DST with the CRF stimulation test. Patients undergoing this test are first administered a single dose of dexamethasone at midnight and the following day receive an intravenous injection of a low dose of CRF. The amount of ACTH and Cort released is much higher among individuals that suffer with depression (Holsboer et al., 1995). The DST and dex/CRF test have different results most likely because the dose of CRF can enter the CNS and activate CRFR1, while dexamethasone cannot enter the CNS and thus cannot activate CRFR1. In fact, the study proposes that it can detect depression with an accuracy of 80% (Heuser et al., 1994).

The HPA-axis is very sensitive to early stress exposure. Experiments that stressed pregnant female mice showed that the pups had increased HPA-axis activity throughout adulthood. On the other hand, very brief daily separation of mother and pups results in a reduction of both emotionality and Cort secretion in adulthood (Levine and Mullins, 1966; Reul et al., 1994). It becomes apparent that either a hypo- or hyper-active HPA-axis can manifest as psychiatric illness. While most psychiatric illnesses have been associated with a hyperactive HPA-axis, PTSD has been linked to a hypoactive HPA-axis (Justice et al., 2015).

Additionally, early life stress has been shown to play a role in the adult stress responses. In humans, sustained stress exposure in adolescence from extreme deprivation and abuse has been linked to sensitization of CRFR1 mediated stress responses and exaggerated responses to stressors (Heim et al., 2008). Acute and chronic stress during early life increases the amount of CRF located in the locus coeruleus (LC) noradrenergic system, a member of the CAS system that is vital to the stress response. The activity of LC noradrenergic systems is elevated during times of stress and anxiety in humans. This evidence suggests that overactivation of the CRF network and the HPA-axis, whether during early life or adulthood, are a major driver of psychiatric illnesses such as anxiety and depression.

It has been proposed, although not proven, that antidepressant efficacy is due to normalization of the HPA-axis. This hypothesis was generated after it was observed that in depressed patients cortisol secretion is elevated. Additionally, the number of CRF neurons and CRF concentrations in limbic brain regions is increased; however, the number of CRF binding sites in the cortex is reduced (Holsboer, 2000).

It is important to discuss the behavioral paradigms used to measure depressive-like behavior in mice for future researchers. Previously, I discussed behavioral paradigms that test anxiety-related behavior in mice. Depressive-like behavior in mice is measured mainly by two behavioral paradigms; the forced swim test (FST) and the tail suspension test (TST). In the forced swim test, a mouse is placed in a beaker of water and the length of time it swims before giving up and floats is interpreted as a level of depression. The longer the mouse swims, it is interpreted as less depressive-like behavior (Choi et al., 2013; Vincent and Jacobson, 2014). In

the tail suspension test, a mouse is attached to a hook by the tail and the time it struggles to become free is measured. The time immobile is interpreted as depressive-like behavior where a longer time immobile equates to higher depressive-like behavior (Solich et al., 2015; Vucković et al., 2008).

In summarizing this discussion, it can be concluded that CRF and HPA-axis activity, along with specific brain structures, play a major role in the development of pathological stress-related diseases such as anxiety and depression.

CRF's Potential Role in the Symptoms of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder following Alzheimer's Disease. It has an incident rate of 2% of the adult population over 60 (Dhungel et al., 2015; Massano and Bhatia, 2012). Only 10% of PD cases are linked to genetic causes while the remaining 90% are considered sporadic with unknown origin (Kibel and Drenjančević-Perić, 2008). The disease is generally thought of as a motor disease caused by the death of dopaminergic neurons in the substantia nigra pars compacta (SNc) resulting in a loss of dopaminergic neurotransmission in the STR (Obeso et al., 2014). The motor aspects of the disease are categorized by four distinct features: bradykinesia (slow movement), rest tremor, rigidity, and postural and gait impairments (Massano and Bhatia, 2012). Neuropsychiatric disorders of anxiety and depression are key non-motor symptoms of the disease that aid in diagnosis and are found in 40-50% of PD patients (Massano and Bhatia, 2012; Yamanishi et al., 2013). Surprisingly, the non-motor symptoms of the disease are only recently garnering attention as part of the primary features of PD. Mood swings linked to varying degrees of motor functioning are reported in 66% of PD patients. These aspects can be correlated by the occurrence of decreased mood and high anxiety with poor motor function (Vlastelica, 2011).

Stress can exaggerate the motor deficits in PD. Specifically, chronic stress and the associated neuroendocrine response can produce a neurotoxic effect that can lead to the death of dopaminergic neurons in the SNc (de Pablos et al., 2014; Smith et al., 2002, 2008). Since stress has the potential to cause the loss of dopaminergic neurons and is implicated in the etiology of neuropsychiatric disorders like anxiety and

depression, the potential for a link between the motor and non-motor symptoms of PD emerges through the stress pathways (Krishnan and Nestler, 2008; Nestler et al., 2002). Investigating the circuitry controlling the body's response to stress could provide crucial insights into the pathogenesis of PD and lead to new targets for therapeutics. From the evidence presented in this study, I propose this link to be the GPe.

GPe function is greatly disrupted in the dopamine depleted brain. In a 6-OHDA mouse model of PD, dopamine depletion was shown to decrease autonomous activity in Npas1 neurons, while PV+/prototypic neuron firing is preserved (Hernandez et al., 2015). In my behavioral study, I have shown that CRFR1 expressing prototypic neuronal activation leads to an increase in anxiety-related behavior and an increase in movement in protected regions of the maze. I have shown that activation of Npas1 neurons, which are mostly arkypallidal (all arkypallidal neurons are Npas1+), leads to a reduction in anxiety-related behavior and decreased movement in the protected regions of the maze. Interpreting my results in the context of a PD model, a decrease in the activity of Npas1 neurons could drive anxiety by allowing CRF activation of CRFR1 expressing GPe neurons to proceed without the counterbalancing effect of Npas1 neuronal connections to the STR. This suggest that anxiety-related behavior mediated by the GPe can be "selected" based on which neuronal subtype is firing and that there is a mechanism of balance between the two neuronal subtypes, similar to the direct and indirect pathway model. Activation of CRFR1 neurons increases movement via the direct pathway while activation of Npas1 neurons decreases movement via the indirect pathway. This revised model improves upon the once-held

belief that the GPe is a homogenous nucleus with a single function in the indirect pathway (Albin et al., 1989; Alexander and Crutcher, 1990; Calabresi et al., 2014; Y. Smith et al., 1998; Wichmann and DeLong, 1996). Based on my findings, I predict that GPe neurons in dopamine depleted brains would display increased excitability due to altered striatal GABAergic input, which would manifest as increased anxiety-related behavior. Arkypallidal neurons may decrease their inhibitory input to the STR leading to a decrease in movement similar to what is observed in PD. We are just beginning to understand the Arkypallidal-STR pathway so this may not be the case in PD. As the GPe is highly dysfunctional in PD, any aberrant signaling may lead to both motor and non-motor symptoms as I have shown the GPe is involved in both motor and anxiety-related behavior.

In response to a physiological stressor such as neurodegeneration of dopaminergic neurons in the SNc, the stress response is activated. PVN release of CRF into the GPe resulting in increased movement via activation of the direct pathway counterbalanced by decreased movement controlled by Npas1 neurons in the indirect pathway may lead to such symptoms as tremors (shaking of the hands). As the severity of motor symptoms are correlated to the severity of the non-motor symptoms including depression and anxiety, it is not unreasonable to consider that increased CRF signaling as a result of activation of the stress response accounts for this correlation.

As discussed, the GPe is in a powerful position to exert its influence on all “downstream” nuclei of the basal ganglia. A dysfunctional GPe receiving excitatory CRF input likely precipitates anxiety-related disorders. Also, it is possibile that a

dysfunctional GPe could manifest as elevated arkypallidal activity and a “no stress” response to normal stressors. As I have shown that the GPe has bidirectional control over anxiety-related behavior, we can consider the GPe as an anxiety BG nucleus. Any reduced firing in anxiolytic Npas1 GPe neurons, like the ones experienced in models of PD, is likely to manifest as an increase in anxiety-related behavior, possibly reaching the levels of panic disorder which half of PD patients experience. The question is whether CRF signaling plays a role in this anxiety or does the dysfunction in the GPe alone contribute to the psychiatric symptoms experienced by PD patients. Excessive CRF signaling to the GPe, as part of the stress response activated by the loss of dopaminergic neurons, could manifest not only as anxiety-related behaviors and illnesses. Most PD patients also experience depression. As discussed in the previous section, hypersecretion of CRF is a hallmark of depression. In this way, elevated CRF signaling and GPe dysfunction could lead to both motor and non-motor neuropsychiatric disorders that plague PD patients.

The question arises as to whether an individual with a predisposition to anxiety disorders, either through natural processes like early life stressors or genetic causes such as polymorphisms in CRF receptors, is predisposed to develop PD. Based on known PD progression, it seems likely that an early symptom of PD would be anxiety that gradually increases as dopamine levels decrease. The loss of dopamine would amplify GPe dysfunction as any changes in signaling in the STR will be propagated to the GPe. By identifying the GPe as an anxiety nucleus in the BG that receives input from the stress system in the form of CRF/CRFR1 signaling, the GPe could serve as a link between the psychiatric and motor symptoms of PD.

This body of work may set the stage for new interventions that could address both motor and psychiatric aspects of PD. With further advances in technology, a synthetic peptide CRFR1 antagonist that crosses the blood brain barrier will be generated. I predict that this antagonist would improve the functioning of PD patients by attenuating the deterioration of motor and non-motor behaviors. Considering my hypothesis that PD symptoms are the result of imbalanced prototypic and arkypallidal neuronal activity in the GPe, activating arkypallidal neurons may be a beneficial treatment for anxiety.

It would be interesting to repeat the same DREADD experiments in 6-OHDA PD mouse models to determine how GPe mediated anxiety-related behavior and movement are effected in the absence of striatal dopamine. If an excitatory receptor expressed abundantly in Npas1 neurons is identified, a novel agonist could be generated to activate these neurons to lessen anxiety. In combination with the next generation of CRF antagonist, an Npas1 excitatory agonist acting primarily in the GPe may restore the delicate balance that is disrupted by dopamine depletion. As discussed in the introduction, dopamine depletion directly effects the STR by generating an imbalance in D1 excitatory receptor and D2 inhibitory receptor activation. The STR then propagates this dysfunction “downstream” to the GPe. If we could develop drugs that act on the GPe and/or inputs to the GPe from the STR, we may not be able to stop the progression of PD, but we could improve the quality of life of PD patients dramatically.

Additionally, identifying the GPe as a likely CRF responsive nucleus that has bidirectional control over anxiety-related behavior could lead to the GPe being a target

for therapeutics such as deep brain stimulation (DBS). Due to the heterogeneity of brain regions where DBS electrodes are placed and the relevant target neuronal subtypes *e.g.* prototypic vs arkypallidal neurons, it has been difficult to reveal the underlying mechanisms of DBS, however high-frequency stimulation of the STN is beneficial in the treatment of PD (Gradinaru et al., 2009). It is possible that DBS in the GPe may be effective in treating anxiety, and in addition, the motor symptoms of PD. Experiments in humans need to be performed before we can draw this conclusion

While these proposals will require years of intensive research and decades of drug development, I believe that I have laid the groundwork for future research into the role of the GPe in anxiety and the possibility of generating targeted therapeutics to improve the lives of those suffering from PD.

Anxiety-related Behavior and Movement as GPe Mediated Action Selection

As discussed in the introduction, a recent computer model of the GPe calculates that prototypic and arkypallidal neurons are capable of optimal action selection (Bogacz et al., 2016). Action selection can be reflective (goal-directed behavior) or reflexive (habitual behavior) (Dolan and Dayan, 2013). Goal-directed behavior can be defined as involving active deliberation and adaptive flexibility to a changing environment. On the other hand, habitual behavior can be defined by its automaticity and inflexibility (Dayan, 2009; Dolan and Dayan, 2013).

I observe an increase in anxiety-related behavior and movement when activating CRFR1+/prototypic neurons, and a reduction in the same behaviors when activating Npas1+/arkypallidal neurons. Of the behavioral paradigms, I will consider the OFA in the process of the proposed role of the GPe in action selection. When prototypic neurons (CRFR1-cre) neurons are activated, I observe an increase in anxiety-related behavior as measured by a decrease in the ratio of time spent in the center region versus total time. Also, I observe an increase in the total distance traveled in the surround of the maze, a region that elicits the least anxiety. Since the increase in movement was region specific; I concluded that it was a component of the anxiety-related behavior. While the conventional interpretation of the OFA results clearly demonstrates an increase in anxiety-related behavior, movement in the surround is not a measure of anxiety by convention (Green et al., 2007).

In the classic model of the BG, activating prototypic neurons that project to the STN should result in decreased movement, not increased movement as I observe (Hernández et al., 2015). This suggests that activation of prototypic neurons, in

isolation, have a function not described by the direct/indirect pathway model. Possibly, this discrepancy can be explained by the GPe's proposed role in action selection.

Activation of the stress response by CRF/CRFR1 signaling necessitates action selection in the behaving animal. My data demonstrates that anxiety-related behavior is mediated by prototypic neuron activation in the GPe. In response to a stressor, all actions that are salient to survival should take precedence in BG motor programs. Now that an action must be selected for survival, escape is the reflective/goal-directed behavioral action that may be chosen (Hauger et al., 2009). The opposite action that may be selected is the reflexive/habitual action for the animal to freeze (Botta et al., 2015; Dickinson, 1985; Dickinson and Balleine, 1994; Snyder et al., 1985; Stricker and Zigmond, 1974). This is the classical "flight or fight" dilemma in the initiation of the stress response. Specifically, when faced by a stressor, the animal has two choices. This is action selection where the most salient for survival should be chosen. If there are two choices for the animal to make, escape or freeze, why did all of my mice select to escape?

DREADD activation of prototypic neurons is by definition an artificial manipulation in an artificial system, Cre expressing mice. As the mice were naive to the OFA when tested, the action they selected, escape, was goal-directed behavior and not habitual. Further, escape behavior is a goal-directed behavior because it is intentional and has a beneficial outcome (Dolan and Dayan, 2013). However, I have generated a total BG network disturbance by my artificial manipulation in the GPe. This manipulation must have an effect on all of the "downstream" nuclei in the BG and on the feedback the GPe and output nuclei of the BG receive. As the mice have

experienced anxiety previously, some of the mice should have made the reflexive or habitual action selection and displayed freezing behavior. I propose that artificial activation of prototypic neurons in the GPe impairs the ability of the GPe to make the optimal action selection and the system biases to one choice, escape. My manipulation has disrupted the ability for the GPe to make a choice between reflective or reflexive behaviors in response to the anxiety-eliciting stimulus. Potentially, the network of the BG is biased to the selection of the goal-directed action, and this leads to the increased movement in contradiction to the GPe playing its classical role in the BG indirect pathway.

On the other hand, when I artificially activate arkypallidal neurons as in DREADD activation of Npas1 neurons, I observe less anxiety-related behavior and movement. In the OFA paradigm, the mice do not select either to escape or to freeze. The mice display more center region time and more exploratory behavior, which is indicative of a decrease in anxiety-related behavior. I propose that activation of arkypallidal neurons in the GPe impairs the ability of the BG to select the appropriate action (similar to activation of prototypic neurons). Arkypallidal neurons project to and send a “stop” signal to the STR. Activation of these neurons would modify the normal functioning of the STR which has been shown to play a role in both goal-directed and habitual behaviors (Balleine, 2005; Corbit and Balleine, 2005; Killcross and Coutureau, 2003; White, 1997; Yin et al., 2005, 2004; Yin and Knowlton, 2006). It is not unreasonable to propose that artificial disruption in the STR may render the GPe’s proposed role in optimal action selection dysfunctional, resulting in no action being selected. The mice just explore the maze which my data supports.

While my behavioral paradigms indicate an increase in anxiety-related behavior in the case of activation of prototypic neurons, the movement phenotype I observe in the OFA may be an artifact of an artificial system. However, the phenotype may provide novel evidence that the unmanipulated GPe is capable of optimal action selection because disruption in GPe circuits has a deleterious effect on action selection (Bogacz et al., 2016). *In vivo* electrophysiological recordings of prototypic and arkypallidal neurons in the GPe during DREADD manipulation in paradigms designed to test action selection (reinforcer devaluation paradigms) may provide evidence to support this proposal.

Concluding Remarks

This study provided the first evidence that a BG nucleus, the GPe, has bidirectional control over anxiety-related behavior and movement that can be influenced by CRF/CRFR1 signaling. The two neuronal subtypes I investigated, CRFR1-expressing prototypic neurons and Npas1 neurons, have opposing behavioral outcomes in anxiety-related behavioral paradigms. It would be of interest to test these mice on behavioral paradigms that focus on other aspects of behavior such as learning and memory as action selection moving from goal-directed behavior to habitual behavior is a form of learning (Dolan and Dayan, 2013). Also, it would be of great interest to inhibit one neuronal subtype while activating the other to further investigate the GPe's role in action selection. With the ever growing field of mouse genetics, the technology to do this already exists.

I identified, for the first time, the source of CRF input to the GPe and discovered that CRF neurons in the key stress-nuclei project and synapse in the GPe on both CRFR1 neurons and Npas1 neurons.

In conclusion, this study has provided a foundation that identified the GPe as a BG nucleus that's neuronal subtypes have bidirectional control over anxiety-related behavior and movement. Future researchers who wish to investigate CRF/CRFR1 signaling in the GPe, or to investigate basal ganglia-mediated anxiety-related behaviors, will hopefully find this body of work beneficial to both the BG and stress fields.

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Vita

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