DISSECTING THE FUNCTIONAL HETEROGENEITY OF SEROTONERGIC SYSTEMS THAT REGULATE FEAR AND PANIC

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DISSECTING THE FUNCTIONAL HETEROGENEITY OF SEROTONERGIC SYSTEMS THAT REGULATE FEAR AND PANIC

Serotonin (5-HT) is heavily implicated in severe anxiety and trauma-related disorders, such as panic and post-traumatic stress disorders. Overall, site-specific pharmacological manipulations show that while 5-HT enhances anxiety-associated/avoidance behaviors in the amygdala, 5-HT inhibits panic-associated escape behaviors in the perifornical hypothalamus region (PeFR). Yet, our understanding of how specific serotonergic networks and co-transmitters regulate these conditions, but also other aspects of innate panic (e.g., cardioexcitation or thermal response that occur during a flight or escape response) or conditioned fear behaviors is still elusive. Therefore, utilizing circuit-based gain- and loss-of-function approaches to selectively manipulate amygdala- and PeFR-projecting serotonergic systems, we hypothesize that specific serotonergic networks projecting to the amygdala and PeFR respectively enhance conditioned fear responses and attenuate innate panic-associated behaviors and physiological responses. There are two main chapters in this dissertation. In Chapter III, retrograde tracing revealed that the amygdala-projecting neurons from dorsal Raphe (DR) were almost exclusively serotonergic (92-95%) concentrated in the dorsal/ventral (DRD/DRV) DR, with few non-serotonergic neurons. While selective lesioning of this network with saporin toxin (SAP) facilitated the extinction of conditioned fear behavior, selective optogenetic activation of amygdala-projecting DRD/DRV cell bodies using intersectional genetics reduced extinction of conditioned fear behavior and enhanced anxiety avoidance. In Chapter IV, retrograde tracing showed that the PeFR was innervated by equally selective serotonergic networks concentrated in the

lateral wings DR (lwDR) and median Raphe (MR). Contrasting with the results from the amygdala-innervating 5-HT system, lesioning the PeFR-projecting serotonergic network from lwDR/MR was accompanied by reduced extinction of conditioned fear behavior, increased anxiety avoidance, and increased CO_2-induced panic (elevated escape responses and enhanced cardioexcitation). Conversely, selective activation of lwDR/MR serotonergic terminals in the PeFR decreased anxiety-associated behaviors; inhibited CO_2-induced panic, and induced unconditioned and conditioned place preferences. The circuit-based approach data presented here show that amygdala- and PeFR-projecting 5-HT neurons comprise distinct circuits underlying opposite roles enhancing anxiety/fear responses in the amygdala and dampening fear/panic responses in the PeFR. The identification of distinct circuits controlling anxiety, fear, and panic responses is a fundamental step towards the development of more effective therapies for psychiatric conditions such as anxiety and trauma-related disorders.

Theodore R. Cummins, PhD, Chair

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List of Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
AAV	Adeno-associated virus
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Anteroposterior
APA	American Psychiatric Association
BA	Basal amygdala
BLA	Basolateral amygdala
BMA	Basomedial amygdala
BNST	Bed nucleus of the stria terminalis
BP	Blood pressure
CAV	Canine adenovirus
CeA	Central amygdala
ChR2	Channelrhodopsin
CO_2	Carbon dioxide
CR	Conditioned response
CRF	Corticotrophin releasing factor
CS	Conditioned stimulus
CTB	Cholera toxin b subunit

DAB	3,3'-diaminobenzidine tetrahydrochloride
DR	Dorsal Raphe nucleus
DRC	Caudal subdivision of dorsal Raphe nucleus
DRD	Dorsal subdivision of dorsal Raphe nucleus
DRR	Rostral subdivision of dorsal Raphe nucleus
DRV	Ventral subdivision of dorsal Raphe nucleus
DSM-V	Diagnostic and Statistical Manual of Mental Disorders fifth edition
DV	Dorsoventral
EPM	Elevated plus-maze
ETM	Elevated T-maze
eYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
GABA	gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
HR	Heart rate
IgG-SAP	Saporin toxin conjugated with immunoglobulin G
IL	Infra-limbic cortex
ITI	Inter-trial interval
LA	Lateral amygdala
LH	Lateral hypothalamus
LSD	Fisher's least significant difference test
LTD	Long-term depression

LTP	Long-term potentiation
lwDR	Lateral wings subdivision of dorsal Raphe nucleus
lwDR/MR→PeF	Perifornical hypothalamus-innervating projections from lateral wings dorsal Raphe and median Raphe nuclei.
lwDR/MR→PeF:ChR2	Perifornical hypothalamus-innervating projections from lateral wings dorsal Raphe and median Raphe nuclei expressing channelrhodopsin
ML	Mediolateral
MR	Median Raphe nucleus
NaLac	Sodium lactate
NIMH	National Institute of Mental Health
NMDA	*N*-methyl-D-aspartate
O_2	Oxygen
OFT	Open field test
OX	Orexin
OXA	Orexin A peptide
PA	Panic attack
PAG	Periaqueductal gray
PB	Phosphate buffer
PBS	Phosphate buffer with 0.9% saline
PBST	Phosphate buffer with 0.9% saline and 0.3% triton X
PD	Panic disorder
PeF	Perifornical hypothalamus

PeFR	Perifornical hypothalamic region
PFC	Prefrontal cortex
Phal-l	Phaseolus vulgaris leucoagglutinin
PTSD	Post-traumatic stress disorder
RDoC	Research Domain Criteria
RTPP/A	Real-time place preference/avoidance
SAP	Saporin toxin
SERT	Serotonin transporter
SERT-SAP	Saporin toxin conjugated with serotonin transporter anti-body
SI	Social interaction
SSRI	Selective serotonin reuptake inhibitor
TPH	Tryptophan hydroxylase
UNC	University of North Carolina
US	Unconditioned stimulus
VLPA	Ventrolateral periaqueductal gray
VMH	Ventromedial hypothalamus
VMHvl	Ventromedial hypothalamus, ventrolateral part
vmPFC	Ventromedial prefrontal cortex

Chapter I: Introduction

1.1. Establishing Operational Criteria to Evaluate Emotion States

The neuroscience field is experiencing an ongoing revolution of molecular tools that are allowing researchers to interrogate the function of neural circuits and their contribution to various physiological and behavioral processes in laboratory animal models. Amongst these processes, perhaps the most controversial and elusive is emotion and behavioral responses that potentially indicate emotional responses. Even though it might seem simple and obvious when one examines emotion introspectively, emotion still remains scientifically challenging to define in objective terms. Although many proposals have failed to reach a consensus definition for emotion, removing this term as suggested by LeDoux (2012) does not solve the problem of how to study and compare the fundamental aspects of animal and human emotions.

From an anthropomorphic perspective, emotions are referred by many psychologists and neurobiologists (Barrett et al., 2007; Barrett and Russell, 1999; Russell, 2003; Salzman and Fusi, 2010; Scherer, 2009) as experiences that lead to the subjectively described "feelings" in humans. Expanding from these authors standpoint, any given internal/external stimuli would lead to a combination of different responses (i.e. cognitive, motivational, somatic, motor, and autonomic) and ultimately converge into an emotional experience that is knowingly voiced by humans as feelings. Yet, with animals only behavior, somatic, and visceromotor responses are measurable. Many argue that since these feelings, such as fear, can only be assessed by verbal report, they cannot be extended to and studied in other non-human mammals due lack of such spoken language. Going further, because of the direct connection between emotional response and feelings described above, it has

been proposed that emotions are also anthropomorphic experiences and could not be studied in any other animal other than humans (LeDoux, 2012).

However, what if emotions were cause, instead of merely consequences as described above, of externally observable behaviors, cognitive, somatic, and autonomic responses, as proposed by Anderson and Adolphs (2014)? This was first recognized by Darwin (1872) in his assay about *The Expression of the Emotions in Man and Animals*. He suggested that some physical manifestations of emotional expressions are shared from a functional and evolutionary perspective and could be easily recognized across mammal species, as illustrated in **Figure 1**. However, Darwin did not provide an operational criteria for identifying the emotional expressions described in his work other than his own intuitions. Providing consistent, operational criteria is essential when suggesting that emotions are a conserved feature across the species.

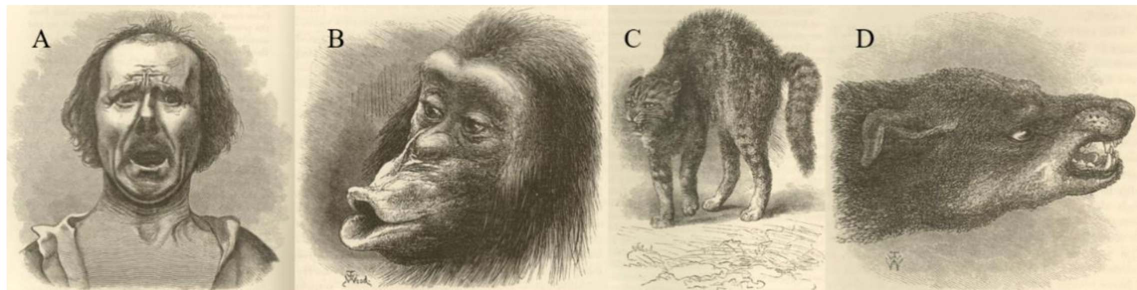

Figure 1 – Emotional expressions across species. Fear expression in a human (A), chimpanzee showing disappointment (B), and hostility in a cat (C) and a dog (D). From Darwin (1872).

From Anderson and Adolphs' perspective,

> "…emotion constitutes an internal, central (as in central nervous system) state, which is triggered by specific stimuli (extrinsic or intrinsic to the organism). This state is encoded by the activity of particular neural circuits that give rise, in a causal sense, to externally observable behaviors, as well as to associated cognitive, somatic, and physiological responses" (Anderson and Adolphs, 2014).

This perspective is illustrated in **Figure 2**.

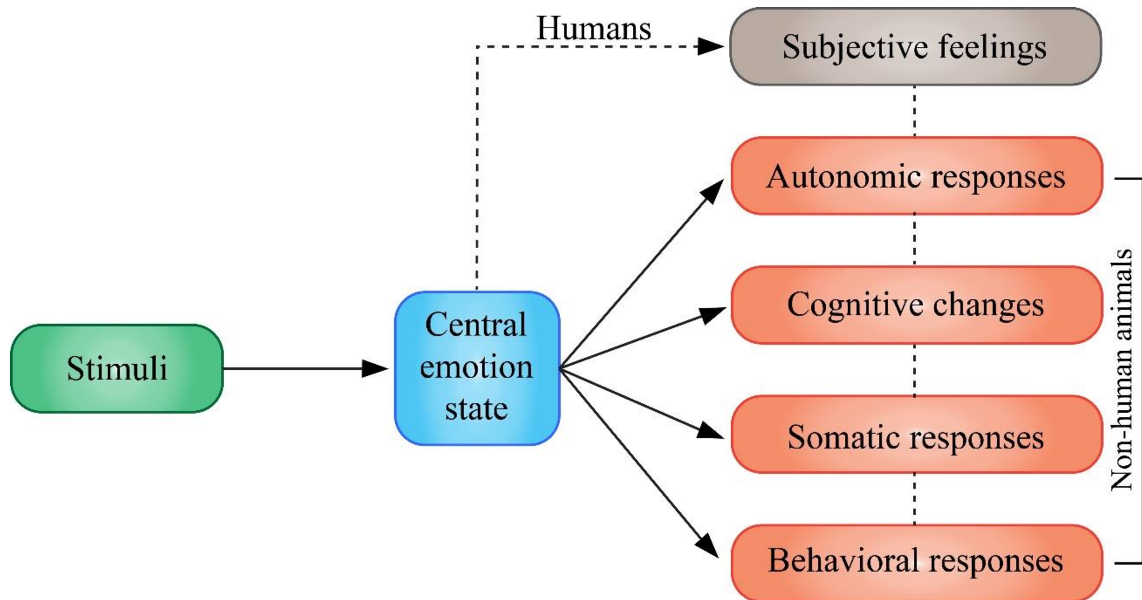

Figure 2 – Model of the causal relationship between emotions and behavior. Stimuli (exteroceptive and interoceptive) perceived by the subject induces activation of specific neural circuits (central emotion state), which in turn causes multiple parallel responses, including subjective feelings in humans. Note that in this model, central emotion states produce subjective feelings in parallel with behavioral and somatic responses in humans. Modified from Anderson and Adolphs (2014).

Because species as whole evolve through the course of generations, so do their brains and neuronal circuits. Therefore, there may be emotional states that are unique to humans, such as social or moral emotions underlying pride and embarrassment (Tangney et al., 2007). The focus here, however, are primitive emotional states (e.g. fear, panic, reward, anxiety, amongst others) elicited by conserved neuronal circuits and expressed by measurable behaviors and physiological changes, rather than idiosyncratic emotions that may be particular to one or other species with higher complexity in the phylogeny tree.

In an attempt to provide operant criteria to delineate central emotions, Anderson and Adolphs laid out four different features that are common to different emotional responses across species, namely: scalability, valence, generalization, and persistence (Anderson and Adolphs, 2014). Even though scalability mean the intensity of a given re-

sponse, a good example for the first feature would also be the qualitatively different defensive responses of a mouse (prey) when facing a rat (its natural predator). At a safe distance, the mouse displays freezing behavior so it can avoid being detected, but as the rat gets closer, this freezing behavior qualitatively shifts into flight or, as a last resource, fight to avoid predation (Blanchard et al., 1998). Moving on, while valence would simply state to the value (positive or negative) of a given emotion, the generalization allows the individual to display the same response over different stimuli across different environments, such as the responses observed in the classical Pavlovian fear conditioning (Davis, 1992). In this case, repeated pairings between conditioned neutral (tone) and unconditioned aversive (shock) stimuli can evoke a measurable behavior (freezing) even after unpaired tones presentations. The last feature, persistence, is in regards to the fact that emotional responses typically outlast the stimuli that evoked them. Using the conditioned response example given above, the freezing or risk assessment behaviors observed in mice typically remain for a while even when the aversive stimulus goes away.

Utilizing the definition of emotions as a type of central neural state elicited by sensory stimuli that in turn control various behavioral, cognitive, somatic, motor and autonomic responses (**Figure 2**), we can objectively explore evidences of emotional states in animal models by directly manipulating specific neuronal circuits of interest. Even though each correlate of primitive emotional states observed in less complex animals very likely differ from those described by humans, there is still a significant overlap between the neural circuits underlying the emotional states. Therefore, there will be instances in this dissertation where I will refer to the collection of responses observed in rats with the terms often used to describe human emotions, such as anxiety (i.e., worry or apprehension), fear to a

potential imminent threat, or panic to an imminent threat. This should not be taken as conceptual mistake, carelessness, nor interpreted as an attempt to anthropomorphize rodents' behavior, but rather as a reference to primitive emotions shared across the species to deal with different temporal or distal threats.

1.2. The Predatory Imminence Continuum Theory

In an ethological context, threats are typically situations where an encounter between prey and predator may occur depending on the distance between these two. It was the ethological perspective of life-threatening encounters between prey and predators that led Robert Bolles to start developing the "Predatory imminence" or "predatory imminence continuum" theory (Bolles, 1970). This theory was modified over the years to adjust to empirical observations and criticism (Bolles and Riley, 1973; Fanselow et al., 1987), and it was later seen as a collection of hierarchically organized behavioral responses, also referred as defensive responses, according to the level of threat that the animal was perceiving to be in (Fanselow and Lester, 1988; Fanselow, 1994). Based on factors such as the distance and likelihood of meeting a predator and routes for escape, three general stages were proposed based on behavioral observation: pre-encounter, post-encounter, and circa-strike (Fanselow and Lester, 1988; Fanselow, 1986).

The first stage, pre-encounter, is typically observed when the animal is foraging before a predator has been identified. Experimentally, some argue that shocks can be commonly used as an aversive stimulus that can represent the risk of a predator. Therefore, by varying the amount of shocks given to rats prior to offering food, one can manipulate the perceived predatory imminence (Fanselow and Lester, 1988; Fanselow et al., 1988;

Helmstetter and Fanselow, 1993). Depending on the predatory imminence, the animal can display increased vigilance and arousal, as well as different foraging patterns. For instance, when a perceived threat is low (none to only a few shocks), rats tend to spend more time foraging and consuming food while being exposed at the food source. On the other hand, when the threat increases (several shocks) rats may decrease time spent foraging at the source while bringing a significant amount of meals to the safety of a nest (Fanselow and Lester, 1988; Fanselow et al., 1988; Helmstetter and Fanselow, 1993).

The second stage, post-encounter, is activated when the threat is identified at a distance. Here, the behavioral responses will directly depend not only on the species, but also on the type and distance that the threat is from the animal. Threatening stimuli cause ongoing behaviors to cease, and the most common observed behaviors encompass freezing, fleeing, or threatening (Blanchard et al., 2011; Bolles, 1970). In rats, however, freezing is typically seen even when escape routes are available (Blanchard et al., 2011; Fanselow and Lester, 1988; Fanselow, 1986). Freezing is a passive defensive response and even though it might look simple, it is a powerful avoidance strategy that reduces motion and visibility, thus decreasing the likelihood the prey will be detected by the predator. Freezing can decrease threat imminence since predators typically use motion to keep track of their prey (Suarez and Gallup, 1981). On a side note, contextual conditioning may occur during subsequent encounters with the predator in the same context, which in turn can evoke freezing responses even in the absence of the threat (Canteras, 2003).

The last stage is circa-strike, which initiates when the predator has started the pursuit towards the prey and is ready to strike. In this case, the predator imminence is the highest, and the typical active defensive behavior observed in rats is fleeing (e.g. jumping

and running) if escape is available, but also vocalization and, less frequently, fighting if there is no escape (Blanchard et al., 1998; Fanselow and Lester, 1988; Fanselow, 1986).

1.3. Anxiety, Fear, and Panic in Rodents and the Predatory Imminence Continuum Hypothesis

The predatory imminence continuum hypothesis explained above encompasses a group of orchestrated behavioral responses that can help illustrate the concepts of anxiety, fear, and panic. Depending on the source, intensity, and duration, intero and exteroceptive stimuli can initiate specific sets of measurable behavioral (e.g. passive versus active defensive responses), physiological, hormonal, and autonomic responses. Example of these responses are increased heart rate (HR), stress hormone secretion, restlessness, vigilance, avoidance of potentially dangerous environments, freezing, and decreased feeding/exploratory behavior, and fleeing responses. Collectively these terms are often used to infer anxiety, fear, or panic in pre-clinical research (Anderson and Adolphs, 2014; Davis et al., 2010; LeDoux, 2014, 2000).

Past research emphasized that these emotional states are particularly controlled by well-defined synaptic and neuromodulatory processes within identified brain regions. However, emerging evidences indicate that these emotional states correspond to the functional states of defined neuronal circuits not only within, but also between various brain regions that have been implicated in anxiety, fear, and panic. The neural basis of these emotional states will be discussed in the section 1.5 below.

Amongst those three emotional states, perhaps the distinction between anxiety and fear is the most tenuous, yet they differ in terms of key dimensions. In particular, anxiety

is often evoked by less predictable and specific threats, or even by specific threats that are physically distant and do not pose immediate risk to the bearer. Typically associated with increased vigilance and arousal, anxiety is best described as a future oriented emotional state encompassing a set of mild measurable behavioral, physiological, hormonal, and autonomic responses that are triggered by potential or distal threats. Consequently, anxiety is a more long-lasting emotional state (Davis et al., 2010) that can be applied to situations similar to those described in the first stage (pre-encounter) of the predatory imminence continuum, often evoking avoidance and increased vigilance. On the other hand, fear is generally an adaptable emotional state measurable by moderate levels of behavioral, physiological, hormonal, and autonomic responses to tangible threats that are detected or evoked by sensorial stimuli. Fear is able to begin and dissipate rapidly according to the duration of the threat presentation (Davis et al., 2010), events similar to those observed in the post-encounter stage. Here, the signature features are freezing, moderate cardioexcitation, and stress hormone response. Lastly, panic, frequently referred to "fight-or-flight" responses, is unquestionably the most intense emotional state of the three. It leads to intense activation of the responses described above due imminent confrontation (circa-strike) with a predator or conspecific confrontations in situations that pose a life-threatening danger (Blanchard and Blanchard, 1990; Blanchard et al., 1998). **Figure 3** illustrates the association between anxiety, fear, and panic and pre-encounter (green), post-encounter (brown), and circa-strike (red), respectively.

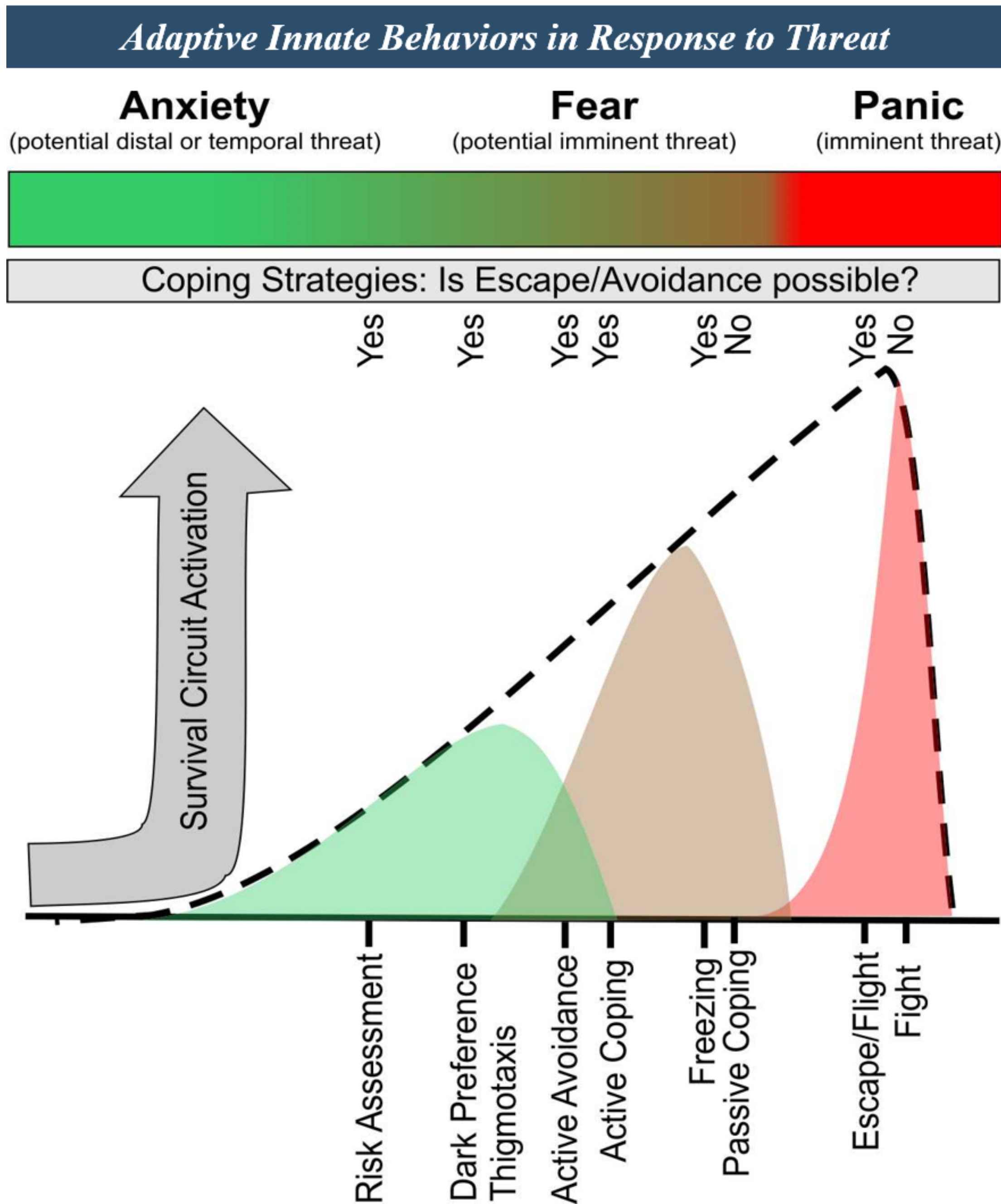

Figure 3 – Adaptive innate behaviors in response to threat. Schematic illustration showing the association of specific behaviors (at the bottom) observed in rodents and emotional states (top) when facing a threat (predator). Green, brown, and red colors in the illustration respectively represent pre-encounter, post-encounter, and circa-strike stages described in the text above. Dashed line represents increasing level of threat.

Around the same time the "predatory imminence continuum" theory was proposed, Caroline and Robert Blanchard observed that wild rodents displayed a very similar pattern

of defensive repertoire that depended upon the presence and distance of a predator (Blanchard and Blanchard, 1988, 1989). According to these authors, the behaviors could be organized into three different levels of defense reactions. The first, defined as risk assessment, takes place when there is a potential unidentified danger. Rats would extend their heads out of a tunnel into the open area and make scanning head movements and occasional stretching and approaching before foraging was initiated. The second level would be observed in the presence of a predator in a distance without any immediate danger. Here, rats often displayed freezing, ceasing all previously ongoing behaviors even in the presence of an escape route. Lastly, when the encounter between prey and predator was imminent, flight was always observed whenever an escape route was available, and defensive fight took place as a last resource when contact was inevitable (Blanchard et al., 1993, 1986).

1.4. Anxiety, Fear, and Panic in Humans: Anxiety Disorders

Anxiety, fear, and panic elicit orchestrated defensive behavioral and physiological responses that have evolved to enable the organism to avoid or reduce harm and thus ensure its survival. Given their importance to survival, it is not surprising that these emotional states evoke a similar set of responses across the species. The presence of shared and conserved traits also implies that predatory threat is pervasive and present throughout evolutionary time (Nonacs and Dill, 1993).

A question that arises though is where the boundary lies between evolved function and dysfunction. From the evolutionary perspective, a mechanism is working functionally if the responses it produces maximize survival for the individuals as a population. However, when anxiety, fear, or panic become pathological (i.e. excessive for the current

stressor, present in the absence of a stressor, or leads to significant distress or disability), these emotional states lead to huge suffering and disability that can markedly disrupt family life as well as the life of the sufferer.

According to the Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-V), anxiety disorders are classified as a group of mental conditions characterized by significant feelings of anxiety and fear [American Psychiatric Association and (APA), 2013]. In humans, anxiety can be perceived as worry about future events, whereas fear and panic, much like in animals, are respectively moderate and strong reactions (emotional, physical, and physiological) to current events (APA, 2013). These events can be perceived as external (or exteroceptive, e.g. social situations) or internal (or interoceptive, e.g. bodily sensations) threats.

With a high lifetime prevalence estimate in the United States of approximately 31% (Baxter et al., 2013), anxiety disorders constitute the largest group of mental disorders and are a leading cause of disability in most countries of the western society (Forouzanfar et al., 2016; Haro et al., 2013; Wittchen et al., 2011) that rarely undergo spontaneous remission (less than 23% of the cases) (Bittner et al., 2004; Wittchen et al., 2000). It has been estimated that more than $42 billion dollars a year in the U.S. (Greenberg et al., 1999) and $74 billion euros in Europe (Wittchen et al., 2011) were attributed to indirect costs such as disability for individuals and their families suffering from anxiety disorder.

On the basis of the clinical picture, anxiety disorders can be divided into seven main psychiatric diagnostic subtypes: generalized anxiety disorder, specific phobia, social anxiety disorder, separation anxiety disorder, agoraphobia, panic disorder (PD), and selective mutism. Because anxiety disorders are a diverse group of conditions, different vulnerability

and risk factors have been particularly implicated in promoting dysfunctional anxiety in different conditions. However, female sex, family history of anxiety, and early-life stress are common factors that can predispose or increase the chances of one having any of those anxiety disorders and thus deserve special attention.

1.4.1. Vulnerability and Risk Factors for Anxiety Disorders

Women are almost twice as likely as man to develop any type of anxiety disorders. Even though almost no differences between sexes are observed during childhood, they start developing during this phase and peak at adult stage (Costello et al., 2016; McLean et al., 2011). While the reasons for this increased risk are still debatable, it seems that women differ from men in how conditional fear memories are formed and extinguished (Maeng and Milad, 2015). The plausible candidates for these difference are the gonadal hormones, especially estrogen, which has been shown to be implicated in important processes modulating fear responses, such as neurogenesis, synaptic plasticity, and the expression of receptors that are key substrates for learning and memory (Cover et al., 2014). For instance, augmented levels of estrogen in both female rats and women enhance fear memory 24 hours after fear learning (Hwang et al., 2015). The amygdala is a good candidate to modulate this, since studies in female rats have shown that the central amygdala expresses both estrogen receptors α and β (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001), and site specific injection of estrogen in the same region facilitated fear conditioning in ovariectomized mice (Jasnow et al., 2006).

Another important predisposition to developing anxiety disorders is heritability. It has been shown that children of individuals diagnosed with at least one anxiety disorder

have up to fourfold increased risk for developing anxiety disorders at significantly earlier stages in life when compared to children with no family history of anxiety disorders (Lieb et al., 2002). Some valuable insights regarding a considerable genetic overlap between some overall symptoms of different anxiety disorders during adulthood are gathered from monozygotic and dizygotic twins studies (Kendler et al., 2011). These studies also estimate that the heritability of anxiety disorders range between 30% and 40% (Holmes and Singewald, 2013; Kendler, 2001; Shimada-Sugimoto et al., 2015), although some symptoms were not shared and seem to differ mainly on disorders characterized by fear (Kendler et al., 2003). The specific mechanisms of familial transmission and genetic risk loci, however, have yet to be established (Otowa et al., 2016).

Although not applicable to all anxiety disorders, early-life stressful situations such as physical, sexual, and emotional abuse (Afifi et al., 2012; Sareen et al., 2013; Taillieu et al., 2016), as well as parent's divorce (Otowa et al., 2014), have been suggested to act as predictors for subsequent development of such conditions (Vrshek-Schallhorn et al., 2014). More importantly, longitudinal studies have been pointing out that anxiety disorders, with special attention to PD, social anxiety disorder, and agoraphobia are important predictors for the development of depressive disorders and substance abuse (Wittchen et al., 2000). The comorbidity between these conditions is a matter of concern, since it greatly decreases the overall quality of life (Kessler et al., 2009) and leads to an increased mortality rate (Saeed Mirza et al., 2015).

Amongst the psychiatric conditions classified as anxiety disorders, of particular importance to this dissertation are PD, agoraphobia, and post-traumatic stress disorder

(PTSD). An import note is that although PTSD was listed under the anxiety disorders umbrella prior to DSM-V classification, this condition is no longer classified as such, being moved to adjoining chapter of trauma-related disorders in the DSM-V (APA, 2013).

1.4.2. Panic Disorder

Panic Disorder (PD) is a severe anxiety disorder which causes significant disability and is characterized by recurrent episodes of unexpected panic attacks (PAs). Typically, PAs peak within approximately 10 min, have a duration of 25-45 min, and can occur during calm or anxious states (de Beurs et al., 1994; Margraf et al., 1987; Taylor et al., 1986) and the majority of PAs occur without an identifiable trigger (Shulman et al., 1994). Panic attacks are an abrupt surge of four or more of the following psychological and physical symptoms (see **Table 1**), with the most common being pounding heart/tachycardia, dizziness, and paresthesia. With the typical onset of PD occurring in early adulthood (Reed and Wittchen, 1998), the twelve-month and lifetime prevalences estimates of PD in the U.S. are 2.7% (J. et al., 2006) and 4.7% (Kessler et al., 2006, 1994), respectively.

Approximately 50% of PAs in PD patients are considered to be spontaneous since they occur in relatively non-threatening and non-agoraphobic contexts (de Beurs et al., 1994). A remarkable feature of the majority of individuals (60-65%) suffering from PD is that PAs can be precipitated upon hypertonic (0.5 M) sodium lactate (NaLac) infusions (Liebowitz et al., 1985, 1984), or breathing air with higher (>5%) carbon dioxide (CO_2) concentrations (Gorman et al., 1994). These challenges typically do not elicit PAs in healthy controls. Therefore, the initial pathology in PD patients can be conceptualized as

an alteration in the central neural pathways such that a maladaptive panic reaction is initiated in response to a non-life threatening viscerosensory stimuli. This could result in PD patients becoming susceptible to 'unexpected' panic symptoms when exposed to mild viscerosensory stressors (Vickers and McNally, 2005).

Table 1 – DSM-V criteria for panic attack (APA, 2013).

A discrete period of intense fear or discomfort, in which four (or more) of the following symptoms developed abruptly and reached a peak within 10 min.
1. Palpitations, pounding heart, or accelerated heart rate
2. Sweating
3. Trembling or shaking
4. Sensations of shortness of breath or smothering
5. A feeling of choking
6. Chest pain or discomfort
7. Nausea or abdominal distress
8. Feeling dizzy, unsteady, lightheaded, or faint
9. Feelings of unreality (derealization) or being detached from oneself (depersonalization)
10. Fear of losing control or going crazy
11. Fear of dying
12. Numbness or tingling sensations (paresthesia)
13. Chills or hot flushes

A remarkable feature of both naturally occurring and experimentally induced PAs is that they are often followed by respiratory alterations (see **Table 1**) or sensation of suffocation (Sinha et al., 2000). Indeed, there are reports showing a high comorbidity between PD patients and several chronic obstructive pulmonary diseases (Simon and Fischmann, 2005). Along those lines, it was proposed that PAs were mostly due respiratory issues as seen in the suffocation theory of panic, in which difficulty of breathing would be the trigger to the intense fear often accompanying the attack (Ley, 1992). Expanding from this, Donald Klein proposed that PAs would be triggered by a dysfunction in the so-called suffocation alarm system. According to Klein, PD patients would be more sensitive to slight elevations

of physiological parameters such as arteriole partial pressure of CO_2 or lactate levels capable of triggering the dysfunctional suffocation alarm system, thus precipitating a PA (Klein, 1993).

Conversely, expected PAs differ from the unexpected counterpart in that there is an external cue where unexpected PAs have occurred and that is now associated with the induction of the attacks (Shulman et al., 1994). The expected PAs also occur in more stressful situations, such as work or driving, but the remaining episodes typically occur in agoraphobic situations where the person believes that escape might be difficult, or help might not be available. Example of these are public transportation, being in open or enclosed spaces, being in a crowd, or being outside of the home alone (APA, 2013; de Beurs et al., 1994). This argued to be a development of cognitive bias toward threat perception (McNally et al., 1990) and conditioning to cues where subjects experienced unexpected PAs (Van Den Heuvel et al., 2005).

It is at late teenage/early adult stage when major plasticity events take place in the prefrontal cortex (PFC) (Huttenlocher, 1984, 1979; Insel, 2010; Spear, 2000), and there are consistent decreases in volumes of this structure in PD subjects (Asami et al., 2009; Lai et al., 2010; Protopopescu et al., 2006). This helps explain why psychological therapies that strengthen PFC activity, such as cognitive behavioral therapy, are the most effective treatments for PD (Mitte, 2005). Beyond PD, PAs are also prevalent in patients with PTSD (Jensen et al., 1998), mood and substance abuse disorders (Grant et al., 2006). PD is also a significant predictor of poor health outcomes such as hypertension and strokes (Esler et al., 2008), and increases the likelihood of suicidal behaviors. PD also has a high comorbidity with agoraphobia, which occurs in over half of the patients (Kessler et al., 2006), and nearly

two thirds of such patients are more resistant to traditional therapies and suffer long-term disability (Grant et al., 2006; Roy-Byrne et al., 2006; Skapinakis et al., 2011). In **Figure 4** there is a chart illustrating the summarized main features of PAs.

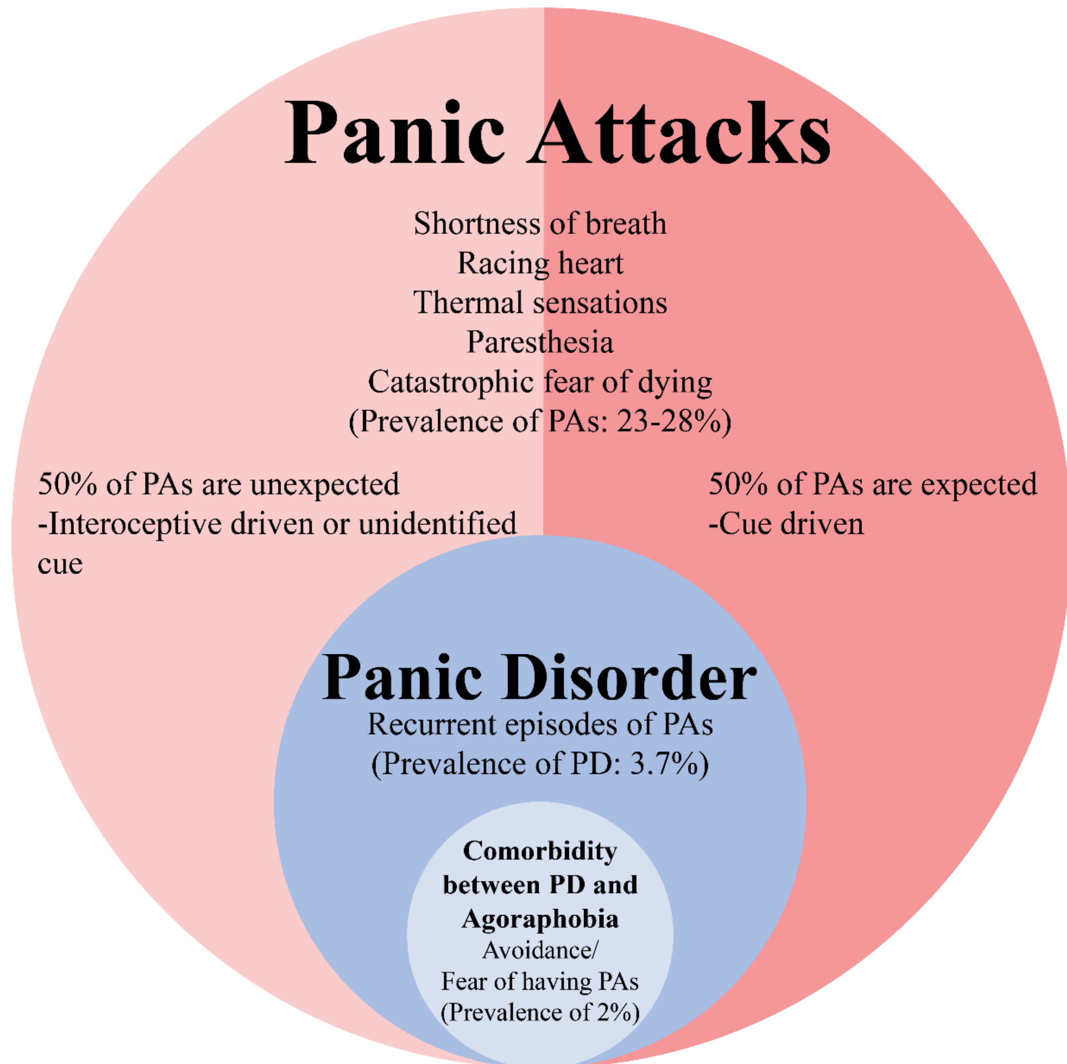

Figure 4 – Chart illustrating the main symptoms and the percentage of expected and unexpected panic attacks (PAs). Note that only a subset of individuals presenting PAs develop panic disorder (PD) and PD with agoraphobia. Adapted from Kessler et al. (2006).

1.4.3. Panic Attacks Are not Limited to Panic Disorder

Initially considered to be an exclusive feature of PD, the most recent DSM-V classified PAs as a specifier to other conditions other than PD. For instance, PAs have also

been observed in patients with mood, psychotic, substance abuse disorders, and other respiratory disease (APA, 2013; Goodwin and Pine, 2002). Moreover, the presence of PAs only are associated with increased suicidal ideation in several psychiatric conditions, such as bipolar disorders and schizophrenia (Frank et al., 2002; Ulas et al., 2007). These observations suggest that PAs can also serve as predictors of greater symptom severity and poorer treatment course when present in a comorbid fashion with other conditions.

1.4.4. Agoraphobia

The diagnoses of PD with or without agoraphobia and agoraphobia without history of PD of versions prior to DSM-V have been replaced by two separate conditions: PD and agoraphobia. This was defended in the review from Wittchen et al. (2010), who suggested agoraphobia to be classified as an independent condition from PD. These authors laid out several reasons to defend their case, and to list a few they emphasized the existence of agoraphobia in communities without symptoms of PAs, epidemiological data showing that PD not always are followed by agoraphobia, agoraphobia only symptoms were associated with outcomes similar to other anxiety disorders, and differences between PD and agoraphobia regarding incidence, gender distribution, and treatment outcome, amongst others (Wittchen et al., 2010).

According to the DSM-V, agoraphobia is described by marked fear or anxiety about exposure, or the anticipation of it, to public spaces such as using public transportation, being in open or enclosed spaces, standing in line, being in a crowd or outside of the home unaccompanied. Patients avoid these situations because in case of panic-like or elevated

fear/anxiety symptoms the subject believes that either help might unavailable or escape might be hindered (APA, 2013).

1.4.5. Research Domain Criteria Approach

An alternative view to the symptom-based classification used by the DSM-V is the Research Domain Criteria (RDoC) approach. The goal of this research framework is not to serve as diagnostic guide, but rather to focus on integrating different levels of information (genomic, neural circuits, behaviors, and self-reports) in order to explore basic functioning of human behavior spanning from normal to abnormal. The RDoC framework is hierarchically organized into *domains*, *constructs*, and *units of analysis*.

While *domain* is a broader term to reflect contemporary knowledge about major systems of emotion, cognition, motivation, and social behavior, *constructs* within each domain define behavioral elements, processes, mechanisms, and responses that comprise different aspects of the overall range of functions. The last, *units of analysis*, are simply a set of several different methods devoted to manipulate and assess molecular, genetic, neurocircuit, and behavioral components of the constructs [National Institute of Mental Health (NIMH), 2019].

Of particular importance for this dissertation is the negative valence systems domain, where lays the responses to aversive situations and contexts, such as fear and anxiety. Within the negative valence systems domain are the constructs defining threats as potential (anxiety), acute (fear), and sustained (escape). Note that these constructs definitions significantly overlap with the pre-encounter, post-encounter, and circa-strike stages of the "predatory imminence continuum" theory discussed in section 1.2.

1.5. Neural Basis of the Emotional States Involving Fear and Panic

In the case of the emotional states involving anxiety, fear, and panic, it is well understood that physiological and behavioral responses are ubiquitously expressed throughout animal phylogeny. Therefore, much of the knowledge regarding the neural basis of these emotional states came from early pre-clinical studies using animal models associated with intracranial stimulation or lesioning of key brain regions to investigate avoidance (anxiety), freezing (fear), and escape (panic) responses in laboratory animals.

1.5.1. The Amygdala Plays a Central Role in Conditioned Fear Responses

As described in section 1.3, fear is generally an adaptable emotional state measurable by moderate levels of behavioral, hormonal, and autonomic responses to tangible threats that are detected by sensorial stimuli or evoked by memories that are associated to an aversive event. The initial observations that the amygdala played a role on fear arose with the study in monkeys with bilateral removal of the temporal lobe (Brown and Schafer, 1888; Klüver and Bucy, 1939), and more specifically lesioning within the amygdala (Aggleton and Passingham, 1981). These monkeys had a striking absence of motor and vocal reactions normally associated with stimuli that evoked fear in non-lesioned monkeys, such as manipulations by the experimenter and presenting monkeys with snakes. Serendipitously, humans with a rare bilateral calcification confined to the amygdala (Urbach-Wiethe disease) showed impaired acquisition of conditioned fear (Klumpers et al., 2015), but also failed to identify the emotion of fear in pictures of human faces, although the identification other emotions, such as happiness and anger, remained intact (Adolphs et al., 1995, 1994).

Early data also showed that the fear-associated freezing responses are tied to conditioned tones paired with unconditioned shock stimulation. For instance, monkeys with amygdala lesions submitted to a conditioned avoidance paradigm presented a lower rate of conditioned fear acquisition when compared to non-lesioned subjects (Weiskrantz, 1956; Weiskrantz and Wilson, 1958). Equally important, these monkeys also showed faster extinction rates during the extinction protocol (absence of shock) (Weiskrantz, 1956; Weiskrantz and Wilson, 1958). The same patterns of fear conditioning and extinction were observed in early studies involving rats (Robinson, 1963) and cats (Brady et al., 1954) with similar lesions in the amygdala.

But the major paradigm of fear conditioning that has been instrumental to uncovering the neural mechanisms of emotional processing has its roots on the early experiments of classical conditioning carried out by Ivan Pavlov. Considered to be the foundation of the conditioned-fear paradigms utilized nowadays, Pavlov paired the presentation of food (unconditioned stimulus, US), at which the dogs would respond by salivating (conditioned response, CR), to the sound of a bell (conditioned stimulus, CS). He later showed that after several CS-US pairings, dogs would salivate upon the presentation of the CS alone (Pavlov, 1910). Based on this experiment, the cued fear conditioning paradigm pairs footshocks (US) to auditory cues (usually tones, CS) to induce a freezing response (CR) in the animals being tested. As previously mentioned, freezing is interpreted as a fear response in rodents since it reduces the chances from being detected by predators (see section 1.1).

In rats, unilateral (LaBar and LeDoux, 1996) or bilateral (Blanchard and Blanchard, 1972; LaBar and LeDoux, 1996; LeDoux et al., 1990; Maren et al., 1996) lesions of the amygdala respectively displayed reduced or blunted conditioned freezing responses to

tone/shock pairings during acquisition or to tone presentation during extinction of fear. Additional deficits in increased HR (Antoniadis, 2000; LeDoux et al., 1990) and corticosterone secretion (Goldstein et al., 1996) have also been observed. Interestingly, patients submitted to unilateral temporal lobectomy had also impaired galvanic skin response during simple and complex fear conditioning tasks (LaBar et al., 1995), suggesting the importance of this brain region in both rodents and humans in mediating fear responses to aversive stimuli.

A question that emerges is how does the amygdala control the array of behavioral, autonomic, and hormonal responses evoked by fear-promoting situations? The answer to this question can be found on the inputs and outputs connecting the amygdala to key brain regions underlying those responses as discussed below.

1.5.2. The Amygdala is an Anatomically Complex Structure that Coordinates Fear Responses with Different Brain Regions

Anatomically, the amygdala is an almond-like shape structure that can be divided into four main nuclei: lateral (LA), basal (BA, which alongside the LA is known as basolateral, BLA), basal medial (BMA), and central (CeA) nuclei. The BLA has a cortex-like structure and is composed of 20% of interneurons expressing gamma-aminobutyric acid (GABA) and 80% glutamatergic principal neurons (Alexander J. McDonald, 1982; McDonald, 1985; Rainnie et al., 2006). While the LA is the main input site for sensory information from sensory cortical and thalamic areas (Burwell et al., 1995; Mcdonald, 1998; Turner and Herkenham, 1991), the BA is rather reciprocally connected with other

brain regions, such as the prefrontal cortex or ventral hippocampus (Hoover and Vertes, 2007; Mcdonald, 1998; Petrovich et al., 2001; Pitkänen et al., 2000; Senn et al., 2014).

A fundamental principle in consolidation of memories associated with aversive events has been attributed to synaptic plasticity in the amygdala complex, more specifically the BLA. This process is known to be mediated by long-term potentiation (LTP) via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors when auditory (e.g. tone) and somatosensory (e.g. shock) stimuli inputs converge into the BLA. LTP has been suggested to be a key cellular mechanism mediating acquisition of CRs during fear conditioning (Nabavi et al., 2014; Quirk et al., 1995; Rogan et al., 1997; Romanski et al., 1993). In line with this are the recent findings showing that optogenetic protocols inducing LTP or long-term depression (LTD) of sensory inputs to the LA were able to respectively produce or inhibit fear conditioned responses post-fear conditioning (Nabavi et al., 2014).

Another important component for the expression of fear responses is the connections made with different brain regions. The somatosensory stimuli arriving in the BLA are conveyed to the CeA, a nucleus formed by GABAergic medium spiny neurons with striatum-like properties that is the major output region of the amygdala (McDonald, 1982). For instance, electrical and chemical stimulations of the CeA have been reported to consistently produce somatic and autonomic components of the fear response which include increases in HR, blood pressure (BP), and freezing (de Molina and Hunsperger, 1962; Hilton and Zbrożyna, 1963; Iwata et al., 1987; Roldán et al., 1974; Ursin and Kaada, 1960). Indeed, electrolytic- or NMDA-induced lesions that included the CeA post-fear conditioning attenuated auditory CS evoked freezing (Goldstein et al., 1996; Goosens and Maren, 2001) and

corticosterone release (Goldstein et al., 1996). These responses can be explained by the direct CeA projections to the hypothalamus, which mediates prominent endocrine and autonomic responses, and the midbrain, more specifically the ventro-lateral (vlPAG) periaqueductal gray (PAG), which mediates motor responses, such as freezing, to fear stimuli (Gray et al., 1989; Krettek and Price, 1978).

Further exploring these projection regions, LeDoux and colleagues observed that while lesions in lateral hypothalamus (LH) prevented elevation in the arterial BP induced by tone (CS) post-shock (US)-CS pairings without affecting freezing responses, caudal PAG lesions disrupted freezing but failed to prevent elevation in arterial pressure (LeDoux et al., 1988). Moreover, Tovote et al. (2016) utilized circuit-based optogenetic to further dissect the CeA-PAG pathway mediating freezing responses. These authors elegantly showed that a group of GABAergic projections from the CeA disinhibited vlPAG excitatory outputs to pre-motor neurons in the magnocellular nucleus of the medulla that controlled freezing responses (Tovote et al., 2016).

However, in addition to understanding the processes by which fear memories are formed and expressed, another important feature is the mechanisms by which safety learning occurs. Fear extinction is not simply forgetting since it requires exposure to the CS in the absence of US instead of simply letting the time goes by. In support of this, studies have shown that fear memories can last from months to years if no extinction protocol is carried out (Gale et al., 2004). Even though fear responses diminish during the process of fear extinction (i.e. when presented with only neutral CS), it is widely accepted that extinction per se is a new type of learning in which distinct networks inhibit fear expression [for

review, see (Myers and Davis, 2007)]. Understanding the mechanisms underlying fear extinction has important clinical implications for treating disorders of fear and anxiety, such as phobias, PTSD, and PD.

One of the main structures implicated in the extinction process is the PFC, more specifically the infra-limbic (IL) cortex (a ventromedial PFC region, vmPFC). A number of studies in rats show CS-related unit activity (Milad and Quirk, 2002) and increased plasticity (Herry and Garcia, 2002; Herry and Mons, 2004; Hugues et al., 2006) post fear extinction, and brief electrical stimulation of IL during extinction training reduced CR and facilitated retention of extinction (Milad et al., 2004; Milad and Quirk, 2002). More importantly, human BOLD signal assessed by fMRI showed increased activity in the subgenual anterior cingulate (a vmPFC region) that was associated with fear extinction during extinction protocol (Phelps et al., 2004).

Interestingly, Amano et al. (2010) found a link between PFC activity and inhibition of CeA output neurons during fear extinction. They observed that PFC activity during extinction potentiated synaptic inputs to GABAergic intercalated cell mass neurons in the amygdala, which in turn project to the CeA (Amano et al., 2010).This increased level of synaptic inhibition in the CeA fear output neurons is suggested to mediate the suppression of CR at various projection sites of the amygdala, such as perifornical hypothalamus (PeF), paraventricular nucleus of the hypothalamus, and vlPAG. Local lesions in these regions respectively reduced blood pressure response, pituitary-adrenal stress hormones release, and freezing responses to the presentation of CS post CS-US pairing (LeDoux et al., 1988;

Morton et al., 1989). This makes the CeA an important target for pharmacological intervention for treating psychiatric conditions whose symptoms rely on resistant extinction to aversive events, such as PTSD.

1.5.3. The Perifornical Hypothalamus Plays a Pivotal Role in Panic Responses

Up to date, two main brain regions have been equally implicated in the genesis of panic responses in both animal models and humans: PAG and perifornical hypothalamus (PeF)/dorsomedial nucleus of the hypothalamus. While there are both clinical and pre-clinical studies supporting the former [for review, see (George et al., 2019; Schenberg, 2010; Schenberg et al., 2014)], this section is going to focus on the implications of the PeF on adaptive and pathological panic.

Adaptive PA is a survival response that occurs to an imminent threat (Stein and Bouwer, 1997). Such attacks can be triggered by either external/exteroceptive (e.g. predator attacks) or internal/interoceptive (e.g. severe hypercapnia that leads to a suffocation sensation) sensory stimuli (Ehlers and Breuer, 1996; Street et al., 1989). The brain regions underlying these responses started being unveiled in the mid-1920s by Cannon and Briton in decorticate cats. These authors found that cats submitted to this procedure had sympathetic responses (e.g., increases in BP, HR, and plasma concentrations of stress hormones) and "sham rage" behavior (e.g., hissing, arching of back and attempts to bite) to non-threatening stimuli (Cannon and Britton, 1925). Following studies showed that "sham rage" was produced by transections of the cat forebrain immediately prior to posterior regions of the hypothalamus, but any transections caudal to this point then suppressed the "sham rage" (both autonomic and somatic) responses (Bard, 1928). This led Philip Bard to conclude

that structures in the forebrain regions exerted some sort of tonic inhibition of "sham rage" responses, which in turn were attributed to the caudal hypothalamus. Indeed, studies decades later showed that retrograde tracers injected into the PeF lead to robust labeling in the IL cortex (Chen and Smith, 2003; Johnson et al., 2008).

Notwithstanding earlier studies of Cannon, Briton, and Bard, it was only later in the 1940s with the seminal work conducted by Hess and Brugger that the PeF emerged as a brain region underlying the "sham rage" responses described above. Hess and Brugger showed that electrical stimulation of discrete regions of the posterior hypothalamus near the PeF of cats evoked strong autonomic and somatic responses (Hess and Brugger, 1943). Later studies conducted in rodents and rabbits showed that electric stimulations of same PeF region (PeFR) evoked similar cardioexcitation, but also tachypnea (Díaz-Casares et al., 2009; Markgraf et al., 1991), decreases in visceral blood flow, and increases in hind limb blood flow (McCabe et al., 1994). Other authors also found agitated running responses that were proportional to stimulus intensity (Duan et al., 1996), but also escape (Olds and Olds, 1962). These responses are remarkably similar to the adaptive panic/defense reactions (e.g., increased BP, piloerection, arching of back).

The hypothalamus is one of the many regions in the brain that is under tonic GABAergic inhibition which occurs primarily through signaling onto ionotropic $GABA_A$ receptors [for review, see (Lee and Maguire, 2014)]. In support of this are studies using pharmacological stimulation of discrete regions of the PeF in rodents showing these sites to be critical for increased physiological (BP, tachycardia, hyperventilation) and behavioral (running and escape) components of the "fight or flight" response. For instance, local injections of $GABA_A$ antagonists in the PeF elicits escape and flight behaviors (Shekhar and

DiMicco, 1987), anxiety behaviors in the operant conflict test (Shekhar et al., 1990), as well as cardiorespiratory responses [e.g., hyperventilation, tachycardia, pressor response, and thermal changes (Anderson and DiMicco, 1990; DiMicco et al., 2002; Shekhar et al., 1990; Shekhar and DiMicco, 1987; Soltis and DiMicco, 1992). Collectively, these reactions encompass the autonomic and behavioral components of the "fight or flight" response, widely associated with panic/defense reactions.

Additional *ex vivo* analyses of markers that indicate cell activity post panicogenic challenges utilized in the clinics have provided further evidences of the involvement of the PeF. For instance, increased signal of the protein product of the immediate early gene cFos has been observed in the PeF post exposure to gas containing normoxic (i.e. 20-21% O_2) 20% CO_2 (Johnson et al., 2011, 2005) and post i.v. infusions of the panicogenic yohimbine, FG-7142 (inverse $GABA_A$ receptor agonist), d-fenfluramine, or caffeine (Li and Rowland, 1993; Singewald et al., 2003; Singewald and Sharp, 2000). Moreover, exposure to ethologically relevant exteroceptive threats, such as predator odor also induced cell activation in the PeF (Dielenberg et al., 2001).

Indeed, the PeF is one of the key neural substrates that induces core symptoms of PAs in humans. For instance, multiple patients submitted to neurosurgery and deep brain stimulation of the PeF reported intense cognitive fear symptoms, such as panic and fear of dying, as well as physiological perturbations, such as tachycardia, increased BP, and hyperventilation (Rasche et al., 2006; Schoenen et al., 2005; Wilent et al., 2011, 2010).

1.5.4. Animal Model of Panic Vulnerability and Implication of Perifornical Hypothalamus Orexin System

Based on the evidences supporting the PeF as one of the key panic generating sites in the brain and the presence of a tonic GABAergic inhibition in the region, Shekhar and colleagues developed a pre-clinical model of panic vulnerability by chronically reducing GABA synthesis in the PeF. More specifically, slow and sustained unilateral infusions of L-allylglycine, a glutamic acid decarboxylase (GAD) inhibitor (enzyme crucial for GABA synthesis), into the PeF increased anxiety behaviors in the elevated plus-maze (EPM), social interaction (SI), and defensive burying tests without affecting baseline cardiovascular/respiratory parameters (Shekhar et al., 2006, 1996; Shekhar and DiMicco, 1987; Shekhar and Keim, 1997). Interestingly, these animals presented panic behavior when challenged with i.v. infusion of 0.5 M sodium lactate or inhalation of low concentration of CO_2, displaying increased general locomotor, rapid increases in BP, and tachypnea (Fitz et al., 2003; Johnson et al., 2010; Johnson et al., 2008; Johnson and Shekhar, 2006; Molosh et al., 2010; Shekhar et al., 2006, 1996; Shekhar and Keim, 2000, 1997). It is noteworthy that these challenges trigger PAs only in PD patients in the clinic (Gorman et al., 1984; Liebowitz et al., 1984). More importantly, classical panicolytic drugs, such as alprazolam, also attenuated the aforementioned symptoms when administered prior to panic provocation (Johnson et al., 2010; Shekhar et al., 2011), like in PD patients (Liebowitz et al., 1986).

Indeed, this panic vulnerability model can be considered as a valid model of panic predisposition since it 1) recapitulates and array of symptoms that are similar to those presented by patients with PD, therefore providing face validity; 2) has prediction validity

once panic-like attacks are precipitated by several panic-inducing agents (e.g. NaLac, yohimbine, and inhalations of CO_2) and are attenuated by therapeutic agents such as alprazolam (Johnson et al., 2010; Shekhar et al., 2011); and 3) has construct validity since stimulation of the PeF has been demonstrated to elicit PA-like behavioral and autonomic responses in animals (Díaz-Casares et al., 2009; Duan et al., 1996; Hess and Brugger, 1943; Markgraf et al., 1991; McCabe et al., 1994; Shekhar and DiMicco, 1987) that are also accompanied by self-reports of panic and fear of dying in humans (Rasche et al., 2006; Schoenen et al., 2005; Wilent et al., 2011, 2010).

Even though the PeF is a critical site underlying panic responses, the PeF is also an extremely diverse region that harbors heterogeneous population of neurons producing a plethora of hormones and neurotransmitters, such as steroid hormones (e.g. pregnanolones oestrogens, androgens) and peptides (e.g. oxytocin, vasopressin, neuropeptide Y, leptin, orexin, melanin concentrating hormone, amongst others) [for review, see (Borrow et al., 2016)]. A question that emerges is what neurochemical system within the PeF is underlying the PAs observed in PD individuals?

An interesting candidate underlying the panic responses within the hypothalamus are the orexin (OX) neuropeptide synthesizing neurons found only in the PeFR and adjacent LH (Peyron et al., 1998b; Thannickal et al., 2000). Initial work showed that loss of function of the OX system was initially attributed to disrupted sleep in dogs (Lin et al., 1999) and mice (Chemelli et al., 1999), and later clinical studies showed that central OX tone and OX neurons were dramatically reduced in narcoleptic patients (Nishino et al., 2000).

However, these OX producing neurons were also found to be critical for inducing anxiety and panic responses in rats. For instance, studies conducted by our lab found that silencing the hypothalamic gene encoding OX or systemic treatment with ORX-1 receptor antagonists blocked the behavioral and autonomic responses induced by NaLac challenge in panic vulnerable rats (Johnson et al., 2010). Moreover, panic-associated behaviors and cardiovascular responses elicited by 20% CO_2 inhalation were attenuated (Bonaventure et al., 2019; Johnson et al., 2015a, 2012c) or even blocked (Molosh et al., unpublished data) upon pharmacological inhibition of the OX1 receptor, but not OX2. More importantly, OX was found to be elevated in the cerebrospinal fluid of human subjects with panic when compared to controls (Johnson et al., 2010). In line with this, the dual OX receptor antagonist suvorexant was found to improve depression and anxiety symptoms in patients suffering from insomnia in a recent clinical trial (Nakamura and Nagamine, 2017). Experiments to test safety, tolerability, and pharmacokinetics of selective OX1 receptor antagonist in healthy subjects have already started (Salvadore et al., 2019) to set the ground for future clinical trial studies with patients suffering from severe anxiety disorders.

1.6. What is the Role of Serotonin in Fear/Panic?

Serotonin is an indoleamine and is synthesized from the amino acid tryptophan in two stages. With the aid of the rate-limiting enzyme tryptophan hydroxylase (TPH), a hydroxyl group is added to tryptophan, converting it into 5-hydroxytryptophan (5-HTP). This is followed by a decarboxylation mediated by the enzyme aromatic L-amino acid decarboxylase, which removes a carboxyl groups from 5-HTP, converting it into 5-hydroxytryptamine (5-HT), also known as serotonin. These reactions occur in two different cells: in

enterochromaffin cells in the intestine (reaction mediated by TPH 1), but also by the great majority of Raphe nuclei neurons in the central nervous system (reaction mediated by TPH 2) (David and Gardier, 2016).

There are seven families of 5-HT receptors that can be divided into a total of 14 known subtypes of 5-HT receptors (**Table 2**Error! Reference source not found.) (Nestler et al., 2014). All 5-HT receptors except 5-HT3, which is a ligand-gated cation channel, are G protein-coupled receptors that can be further organized into three major classes according to their effector mechanisms.

Table 2 – Serotonin Receptors Present in the Central Nervous System.

Family	Subtypes	Central nervous system distribution
$5\text{-}HT_1$	$5\text{-}HT_{1A}$	Hippocampus, amygdala, septum, entorhinal cortex, hypothalamus, Raphe nuclei
	$5\text{-}HT_{1B}$	Substantia nigra, basal ganglia, superior colliculus
	$5\text{-}HT_{1D}$	Substantia nigra, basal ganglia, superior colliculus
	$5\text{-}HT_{1E}$	Not known
	$5\text{-}HT_{1F}$	Cerebral cortex, striatum, hippocampus, olfactory bulb
$5\text{-}HT_2$	$5\text{-}HT_{2A}$	Claustrum, cerebral cortex, olfactory tubercle, striatum, nucleus accumbens
	$5\text{-}HT_{2B}$	Not known
	$5\text{-}HT_{2C}$	Amygdala, choroid plexus, globus pallidus, cerebral cortex, hypothalamus, septum, substantia nigra, spinal cord
$5\text{-}HT_3$	-	Hippocampus, entorhinal cortex, amygdala, nucleus accumbens, solitary tract nerve, trigeminal nerve, motor nucleus of the dorsal vagal nerve, area postrema, spinal cord
$5\text{-}HT_4$	-	Amygdala, hippocampus, striatum, olfactory tubercle, substantia nigra
$5\text{-}HT_5$	$5\text{-}HT_{5A}$	Not known
	$5\text{-}HT_{5B}$	Not known
$5\text{-}HT_6$	-	Not known
$5\text{-}HT_7$	-	Cerebral cortex, septum, thalamus, hypothalamus, amygdala, superior colliculus

Modified from Nestler et al. (2014).

The first one, 5-HT_1 receptors class, is coupled to $G_{i/o}$ that inhibits adenylyl cyclase, decreasing the production of cAMP. While the second class is made of 5-HT_2 receptors coupled to G_q, which activates phospholipase C, the third class encompasses 5-HT_4, 5-HT_6, and 5-HT_7 receptors, which in turn are coupled to G_s that activates the cAMP-dependent pathway (Nestler et al., 2014).

After being released from serotonergic boutons, 5-HT neurotransmission is terminated by clearing 5-HT from the synaptic cleft with the aid of the pre-synaptic transmembrane protein 5-HT transporter (SERT). SERT is dependent on the concentration of potassium, sodium, and chloride ions to remove 5-HT from the cleft back into the terminals. This is a two-purpose process which interrupts 5-HT neurotransmission and also enable its reuse by the presynaptic neuron (Nestler et al., 2014).

Serotonin transporter is the target of many selective 5-HT reuptake inhibitors (SSRIs) and tricyclic antidepressant classes, first line pharmacological treatment options to treat patients with anxiety and mood disorders (Nestler et al., 2014). Interestingly, polymorphisms in the promoter region of the gene coding for SERT has been shown to lead to low functional expression of the SERT on serotonergic terminals and linked to affective disorders (Collier et al., 1996). Exploring this polymorphism, Lesch and colleagues observed that humans had increased anxiety-associated traits (Lesch et al., 1996). Specifically in regards to PAs and PD, while the SERT polymorphism has not been shown to be associated with increased incidence of PAs, it has been shown to be associated with the severity of panic symptoms (Lonsdorf et al., 2009).

Serotonin plays a role in a variety of brain functions, including motor activity, cognition, sleep, appetite, sensory processing, and particularly emotion. Indeed, 5-HT has long

been implicated in the regulation of aversive emotions such as fear and panic as proposed by several authors (Deakin and Graeff, 1991; Lowry et al., 2005).

The first conflicting evidences supporting the role of 5-HT in the modulation of fear and anxiety date to early 1960s with Geller-Seifter conflict test and electrical stimulation of the dorsal PAG (dPAG). The Geller-Seifter paradigm is one of the oldest, yet still robust, assays to study anxiety and is based on an operant conditioning box to train an animal to press a lever for a food reward. However, during the lever pressing an unpleasant electrical shock is paired with food delivery, thus adding a conflict parameter in that the animal must decide whether to receive food while getting a small shock or receive no food at all (Geller and Seifter, 1960). Rats treated with anxiolytic drugs, such as the benzodiazepines chlordiazepoxide and alprazolam (but not other psychoactive drugs), display more lever pressing when compared to controls [for review, see (Bali and Jaggi, 2015)].

This model was first utilized to show the anxiogenic role of 5-HT in behavioral inhibition. For instance, early studies using either systemic (Geller and Blum, 1970; Robichaud and Sledge, 1969) or intra-amygdala injections of the TPH inhibitor para-chlorophenylalanine (Hodges et al., 1987), intra-amygdala deliveries of the partial 5-HT_1 agonist/5-HT_2 receptor antagonist methysergide (Graeff and Schoenfeld, 1970), and the 5-$HT_{2A/C}$ receptor antagonist ketanserin (Petersen and Scheel-Krüger, 1984) increased the amount of lever pressing/pecking, releasing the punished behavior. Conversely, decreased lever pressing was found post intra-amygdala injection of 5-HT or the 5-HT_{1A} receptor agonist 8-OH-DPAT in rats (Hodges et al., 1987). Collectively, these results suggest that 5-HT was anxiogenic in prosencephalic structures such as the amygdala.

On the other hand, results regarding the anxiolytic role of 5-HT started to emerge. Different groups observed that while reduction of 5-HT neurotransmission in the dPAG facilitated what the authors called “fear-like” escape responses elicited by electrical stimulation of the same region, local microinjection of local 5-HT produced anti-aversive effects (Kiser et al., 1978; Kiser and Lebovitz, 1975; Schenberg and Graeff, 1978; Schütz et al., 1985). These results suggested that 5-HT was anxiolytic in the dPAG, contradicting those effects observed in the amygdala.

The conflicting results whether 5-HT promoted or attenuated anxiety/fear was based on a late assumption that both the amygdala and the dPAG were thought to mediate the same emotional state, i.e. anxiety/fear responses. However, following the work of the Blanchards about distal threat evoking freezing and proximal threats eliciting “fight-or-flight” behaviors (Blanchard and Blanchard, 1989, 1971), and based on a limited number of clinical results [see author's comments on Deakin (2013)], Deakin and Graeff proposed an elegant theory about the dual role of 5-HT. These authors suggested that while 5-HT neurotransmission mediated by dorsal Raphe nucleus (DR) projections via the prosencephalic tract to the amygdala would facilitate anxiety/fear (avoidance/freezing) when processing potential/distal threats, the 5-HT neurotransmission from the DR to the dPAG via the periventricular tract projections would restrain unconditioned “fight-or-flight” panic responses to proximal threats (Deakin and Graeff, 1991). Another role of 5-HT neurotransmission on resilience in the hippocampus was also proposed (Deakin and Graeff, 1991), but its implications are beyond the scope of this dissertation and will not be discussed.

According to Deakin and Graeff, the same neurotransmitter would modulate different emotional states, facilitating the expression of anxiety/fear in the amygdala and inhibiting "fight-or-flight" panic in the dPAG (Deakin and Graeff, 1991). On a biological perspective, 5-HT's mechanism of defense would be to prevent futile "fight-or-flight" reactions when threat is uncertain or distal, which would ultimately avoid unnecessary energy expenditure and detection by nearby predators (Graeff, 1991).

1.6.1. Serotonin Modulates Panic Behaviors in the Perifornical Hypothalamus

Surprisingly, however, no credit was given to the interplay between 5-HT and PeF mediating panic or "fight-or-flight" behaviors in the Deakin/Graeff hypothesis (Deakin and Graeff, 1991). Even though these authors recognized that *"human patients when electrically stimulated at the PAG, medial hypothalamus…during the course of stereotaxic brain surgery"* experienced intense fear followed by marked autonomic activation (typical symptoms of PAs), the PAG, alongside the prosencephalic amygdala and PFC, were the protagonists of Deakin/Graeff hypothesis (Deakin and Graeff, 1991). On a side note, the elusive "medial hypothalamus" referenced by these authors encompassed not only the PeF, but also vicinal hypothalamic regions.

This is surprising since early studies in cats suggest that 5-HT modulates defensive behaviors (fight or threatening responses) in the PeF. For instance, oral and systemic injections of the TPH inhibitor para-chlorophenylalanine induced growling, hissing, defensive posturing and increasing attacks on conspecifics and experimenters (Ferguson et al., 1970; MacDonnell et al., 1971), and that pre-treating cats with the non-selective SERT inhibitor imipramine elevated the threshold of electrical stimulation of the PeF necessary

to evoke growling, hissing, and defensive posturing (Dubinsky and Goldberg, 1971). Even though these systemic injections could be affecting any brain region, these effects seem to be mediated at least partially by the PeF since local injections of 5-HT into this region produced placidity and attenuated the defensive reactions induced by chemical (carbachol-induced) (Allikmets, 1974; Gołebiewski and Romaniuk, 1985) and electrical (Allikmets, 1974) stimulations of the PeF. More importantly, these anti-aversive effects were prevented with local PeF injection of methysergide prior to chemical stimulation (Gołebiewski and Romaniuk, 1985). Conversely, lesioning of 5-HT fibers with local injections of 5,7-dihydroxytryptamine (5,7-DHT) in the PeF potentiated the PeF chemical-elicited defensive responses (Gołebiewski and Romaniuk, 1985).

Recent experiments in rats by Zangrossi and colleagues have corroborated and expanded the aforementioned studies in cats. The defensive behaviors (e.g. flight and jumping) elicited by electrical (de Bortoli et al., 2013) or chemical (bicuculline-induced) (Biagioni et al., 2016) stimulations of the PeF were attenuated by local injection of 5-HT_{1A} and 5-HT_{2A} receptor agonists. These results led the authors to suggest that the modulation of escape responses by 5-HT in the PeF happens in a phasic rather than tonic manner (Biagioni et al., 2016; de Bortoli et al., 2013). Additionally, analyzing the escape response from the open arm in the elevated T-maze (ETM), Zangrossi and colleagues found that deep brain stimulation applied to the DR decreased escape responses (Wscieklica et al., 2017), and similarly to the electrical and chemical stimulation of the PeF, local injections of 5-$HT_{1A/2A}$ receptor agonists reduced escape (da Silva et al., 2011; Nascimento et al.,

2014). Even though local PeF administration of 5-HT_{1A} antagonist by its own did not produce any effect on escape, it blocked the anti-escape effects when administered prior to its agonist equivalent (Nascimento et al., 2014).

Parallel studies indicate that the aforementioned behaviors might be mediated, at least in part, by 5-HT modulation of the OX system. Even though the OX-producing neurons have been implicated in anxiety and panic only recently (see section 1.5.4), studies prior to OX discovery (de Lecea et al., 1998; Sakurai et al., 1998) have already correlated reductions of 5-HT in the PeF with increased arousal (Imeri et al., 1994). Following those lines, later unilateral perfusion of 5-HT into the PeF using inverted microdialysis reduced the number of cFos staining and decreased the discharge of OX neurons in vivo (Kumar et al., 2007). Electrophysiology of PeF-containing brain slices showed that these inhibitions are directly mediated by post-synaptic 5-HT_{1A} and $GABA_A$ receptors (Chowdhury and Yamanaka, 2016; Muraki et al., 2004). It remains to be tested whether the $GABA_A$ post-synaptic inhibitory current comes directly from co-release of GABA from serotonergic neurotransmission or indirectly from activation of local GABAergic interneurons presumably via activation of 5-HT_{2A} receptors.

More importantly, even though some aforementioned studies suggest that 5-HT inhibits escape behaviors associated with panic via the 5-HT_{1A} and 5-HT_{2A} receptors (da Silva et al., 2011; Nascimento et al., 2014), it has not been investigated yet other aspects of an integrative panic response, such as cardioexcitation and thermal changes. Therefore, there is still a gap in knowledge in serotonergic regulation of the PeFR in the context of panic-associated behaviors and physiological responses. More importantly, since site specific injections of drugs ignore circuit-specific neurotransmission and its physiological

properties that occur *in vivo*, it remains to be explored what is the contribution of selective manipulation of PeF-projecting 5-HT networks on aversive responses to clinically relevant panic-inducing challenges, such as CO_2 inhalation.

1.6.2. Serotonin Modulates Fear Behaviors in the Amygdala

Two fundamental aspects suggest that 5-HT is involved with anxiety and fear emotional states processing via modulation of amygdala function. First, acute systemic injection of drugs that block 5-HT reuptake in humans enhances amygdala activation to emotional stimuli (Bigos et al., 2008; Murphy et al., 2009) and increases fear-potentiated startle response (Browning et al., 2007; Grillon et al., 2007). Indeed, these drugs were shown to increase extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001) and enhance freezing responses during acquisition and consolidation sessions of conditioned fear behavior in rodents [(Ravinder et al., 2013); for review, see (Burghardt and Bauer, 2013)]. Along those lines, extracellular levels of 5-HT increase in the amygdala during fear conditioning (Zanoveli et al., 2009) and in response to inescapable stress (Amat et al., 1998). Meanwhile, administration of anxiogenic drugs with diverse pharmacological properties, or administering a CS that was previously paired with a US (shock) increases cFos in 5-HT neurons in the DR (Abrams et al., 2005; Spannuth et al., 2011).

Second, genetic variations that reduce membrane expression of SERT (Lesch et al., 1996) influence amygdala response to aversive stimuli. For instance, human subjects with at least one copy of the short allele of the SERT promoter exhibit: 1) greater amygdala neuronal activity in response to fearful face presentation stimuli (Hariri et al., 2002); 2) abnormal levels of anxiety/fear (Katsuragi et al., 1999; Lesch et al., 1996; Melke et al., 2001); and 3) are more likely to acquire conditioned fear responses (Garpenstrand et al.,

2001) compared with individuals with normal copies of the allele. Similarly to humans, mutation of the SERT gene is also implicated with reduced transcription of SERT in rats (Homberg et al., 2007). Rats with complete loss of SERT have high baseline anxiety-associated behaviors (Johnson et al., 2019; Olivier et al., 2008), enhanced acquisition/delayed extinction of fear, and disrupted inhibition within the BLA (Johnson et al., 2019).

The BLA is indeed a critical part of the innate and learned fear network. Serotonergic fibers are dense in the BLA and primarily originate from specific regions within the DR (Hale et al., 2008). These fibers innervate both BLA principal neurons and various types of local GABAergic interneurons (Muller et al., 2007). Therefore, it has been proposed that 5-HT could modulate the relay of CS and US information to BLA. In support of this, electrophysiological recordings show that there is a short-term depression in the BA, a region of the BLA, followed by LTP. These effects are suggested to be mediated 5-HT signaling at different levels. The transient inhibition would be mediated by presynaptic 5-HT_{1A} receptors located on LA axon terminals that project to BA principal neurons. Activation of these receptors were shown to depress glutamate release and decrease BA activity (Cheng et al., 1998). A second level of the transient inhibition would come from activation of local BA GABAergic interneurons expressing 5-HT_{2A} receptors (Jiang et al., 2009; McDonald and Mascagni, 2007; Rainnie, 1999; Yamamoto et al., 2012). On the other hand, 5-HT also promotes long-lasting facilitation of synaptic transmission in the BA. This is mediated by NMDA-dependent mechanisms upon activation of 5-HT_{2C} receptors in pyramidal neurons (Chen et al., 2003), but also by LTP of BA neurons via activation of 5-HT_4 receptor activation (Huang and Kandel, 2007).

More importantly, the prolonged increase in extracellular 5-HT concentration within the amygdala following stress may contribute to a net loss of local GABA inhibition and subsequent increase in excitation of glutamatergic projection neurons. In support of this is that stress can downregulate the 5-HT_{2A} receptor and reduce 5HT's effects on local GABAergic tone (Jiang et al., 2009), but also enhance cell surface expression of 5-HT_{2C} receptors in the amygdala (Baratta et al., 2016). The combination of these factors under stressful conditions may bias 5-HT neurotransmission toward excitation of 5-HT_{2C} receptors on fear-promoting BLA glutamatergic projection neurons, ultimately enhancing fear-associated behaviors. Overall, these studies suggest that serotonergic projections to the BLA play a role in the modulation of anxiety-like behavioral responses and conditioned fear behavior.

An important note is that these studies have used bath application of 5-HT which may activate more extrasynaptic receptors and not necessarily recapitulated the physiological mechanisms of 5-HT neurotransmission (Unal et al., 2015). To shed light into these mechanisms, Sengupta et al. (2017) used optogenetic stimulation of serotonergic axon terminals in the BA. These authors showed that while low frequency stimulation (≤1 Hz) of 5-HT terminals in the BA evokes glutamate-mediated excitation of local interneurons, high frequency stimulation (10-20 Hz) elicits 5-HT-mediated excitation and/or inhibition of the same neurons via 5-HT_{2A} and 5-HT_{1A} receptors, respectively (Sengupta et al., 2017). This complex pattern most likely reflects the diversity of GABAergic interneurons present in the BLA [for review, see (Capogna, 2014)]. Furthermore, specific stimuli may selectively activate particular serotonergic networks in a behaving rodents, which in turn could promote differential activation of 5-HT networks in the LA, BA, or CeA, or even in specific

neuronal populations within these nuclei. However, a circuit-based approach to selectively activating amygdala-projecting 5-HT networks on anxiety/fear is still lacking.

1.6.3. Anatomical, Functional, and Molecular Heterogeneity of the Dorsal Raphe Nucleus

The first evidence of serotonergic neurons in the Raphe nuclei arose with the work of Dahlström and Fuxe (1964a). Based on the anatomic distribution along the Raphe, these authors proposed in a following paper that the serotonergic neurons should be grouped into 9 distinct nuclei, B1-B9, according to their rostro-caudal organization (Dahlström and Fuxe, 1964b). Their initial nomenclature followed the proposal of Olszewski and Baxter (1954), but nowadays the Raphe nuclei are recognized as nucleus Raphe pallidus (B1), nucleus Raphe obscurus (B2), the nucleus Raphe magnus (B3), B4 is located in the floor of the fourth ventricle (no specific nomenclature was given), pontine Raphe nucleus (B5), dorsal Raphe nucleus (B6-B7), median Raphe nucleus (B8), and caudal linear nucleus (B9). None of these nuclei is exclusively serotoninergic. Of importance to this dissertation are the DR and median Raphe nucleus (MR).

While both DR and MR are brainstem structures, the DR is localized below the cerebral aqueduct and the MR is adjacent to the pontine reticular formation and below the DR. In the mouse brain, the DR and MR contains about 35% and 8% of 5-HT-producing neurons, respectively, and are the major source of serotonergic innervation to forebrain structures, such as hypothalamus and amygdala (Azmitia and Segal, 1978; Ishimura et al., 1988; Muzerelle et al., 2016; Vertes, 1991).

Initially controversial, the idea that the serotonergic neurons were topographically organized in subnuclei within the DR, with unique afferents, efferents, and functional properties, has since become dogma. Evidences gathered over the last two decades support the view that the DR is not a homogenous structure, but rather a diverse nucleus with different subpopulations of both 5-HT and non-5-HT neurons with distinct molecular signature, morphology, and function (Calizo et al., 2011; Clark et al., 2006; Commons, 2015; Fernandez et al., 2015; Gaspar and Lillesaar, 2012; Hale and Lowry, 2011; Kiyasova et al., 2011; Soiza-Reilly and Commons, 2014).

Topographically, the DR is further divided into five subregions based on the morphology and neuronal density, namely: rostral (DRR), caudal (DRC), ventral (DRV), lateral wings (lwDR), and dorsal (DRD) [(Baker et al., 1990; Steinbusch, 1981), for an extensive review of the anatomical organization, see (Hale and Lowry, 2011)]. Conversely, the MR seems a much less diverse structure, with less heterogeneity of gene expression and no apparent anatomical subdivisions (Clark et al., 2006).

An extensive list of studies have characterized efferent projections originating from the DR [for review, see (Waselus et al., 2011)], but it was Jacobs and colleagues who initially showed that injection of a retrograde tracer into the amygdala and several other different regions lead to a different pattern of labeled neurons within the DR (Jacobs et al., 1978). Following those lines, anterograde tracer injection into the rostral and caudal midline DR led to a distinct pattern of innervation between the amygdala/basal ganglia and hypothalamus/PAG/hippocampus, respectively (Imai et al., 1986; Vertes, 1991). Recent anatomical studies using conditional viral tracer and mouse lines expressing Cre-recombinase in SERT neurons revealed that while most of the serotonergic neurons innervating

the hypothalamus originated from the lwDR and the MR, the amygdala is mainly innervated by the midline DR (Muzerelle et al., 2016).

The functional organization of the DR gained strength with the use of the indirect marker of neuronal activity cFos (Dragunow and Faull, 1989). Building evidence showed that aversive stimuli, such as inescapable footshocks, are able to induce cFos activity in various regions of DR (Grahn et al., 1999). In the same vein, intraperitoneal injections of anxiogenic drugs, such as caffeine, the 5-HT_{2C} agonist mCPP, and the $GABA_A$ inverse agonist FG-7142 (Abrams et al., 2005) and local amygdala or intracerebroventricular microinjections of corticotrophin releasing factor (CRF)1 (Spiga et al., 2006) or CRF2 receptor agonists (Staub et al., 2005), induced cFos activation of specific subdivisions of the DR, particularly the DRD/DRV regions. Conversely, exposure to panicogenic stimuli, such as CO_2 inhalation (Johnson et al., 2005), i.v. infusion of sodium lactate (Johnson et al., 2008), and exposure to unescapable stressors such as forced swimming test (Roche et al., 2003) preferably activated lwDR subdivision of DR, but not the MR. Interestingly, activation of the latter region was linked to anxiety-associated tasks, such as the inhibitory avoidance in the ETM test (Spiacci et al., 2012).

Collectively, the aforementioned evidences support the hypothesis proposed by Deakin/Graeff about the dual role of 5-HT on anxiety and panic (Deakin and Graeff, 1991). According to Lowry and colleagues, while serotonergic neurons in the DRD/DRV subdivisions of the DRs project to forebrain limbic regions involved in stress/conflict anxiety-related processes, serotonergic neurotransmission originating from the lwDR of the DR provide inhibitory tone over brain regions controlling fight-or-flight responses. The MR, however, was suggested to be implicated in coping with aversive stimuli and it could be

important for mood disorders like depression [for review, see (Paul et al., 2014; Paul and Lowry, 2013)]. This is interesting because the MR innervate the panic-associated OX system (Sakurai et al., 2005), and these serotonergic inputs strongly hyperpolarizes OX neurons through postsynaptic 5-HT_{1A} receptors (Chowdhury and Yamanaka, 2016; Muraki et al., 2004).

As discussed above, a variety of studies with systemic and site-specific injections of 5-HT receptor agonists/antagonists proposed to investigate how 5-HT is involved in the modulation of anxiety, fear, and panic responses. Even though these studies can provide important insights about the overall role of 5-HT in these emotional states, site-specific pharmacological microinjections ignore circuit-specific neurotransmission and its physiological properties that occur *in vivo*. One way to circumvent this is the use of opto or chemogenetic techniques in combination with circuit-based manipulations. However, very few studies proposed to investigate the role of 5-HT using a circuit-based approach to question the serotonergic systems' role in anxiety, fear, and panic. For instance, targeting DR subpopulations by using optogenetic activation of serotoninergic terminals in the bed nucleus of the stria terminalis (BNST), Marcinkiewcz and colleagues observed that 5-HT enhanced fear and anxiety by activating a subpopulation of local CRF neurons that silenced anxiolytic BNST outputs to the LH (Marcinkiewcz et al., 2016). Following those lines, while activation CeA-projecting DR circuits increased overall freezing time to the conditioned tone in fear learning and memory recall, specific TPH depletion in the same circuit did not affect those freezing parameters (Ren et al., 2018).

1.7. Goals of the Dissertation

It has been almost 30 years since Deakin/Graeff proposed their influential hypothesis of the dual role of 5-HT promoting anxiety in the amygdala and inhibiting panic in the PAG (Deakin and Graeff, 1991). However, the efforts to study the role of 5-HT modulating anxiety, fear, and panic emotional states have relied mostly on behavioral responses and have rarely assessed autonomic parameters (e.g. cardiovascular excitation). These parameters are a pronounced feature of those emotional states, especially panic (see sections 1.4.3 and 1.4.2). Moreover, even though most studies have focused on conventional chemical/electrical stimulation and intracranial pharmacological approaches to question the role of 5-HT modulating anxiety, fear, and panic, there is still a gap in knowledge of what would be the role of specific 5-HT *networks* in these conditions as the conventional approaches ignore circuit-specific neurotransmission and its physiological properties that occur *in vivo*. Furthermore, even though early evidence pointed towards the involvement of 5-HT modulating panic responses in the PeF, this region was largely overlooked in Deakin/Graeff hypothesis (see sections 1.5.3 and 1.6.1).

Taking in consideration the literature discussed above, our hypothesis is that there are independent 5-HT *networks* that facilitate fear responses in the amygdala, but inhibit panic in the PeF. Therefore, the overarching goals of this dissertation are to dissect the contribution of 5-HT *networks* modulating fear and panic responses. In order to do this, I will 1) inject retrograde tracer into the amygdala or PeF to determine which 5-HT systems innervate these regions; and 2) individually manipulate the amygdala- and PeF-projecting 5-HT networks using gain- and loss-of-function approaches in relevant animal models to establish the role of these networks modulating anxiety, fear, and panic emotional states.

1.7.1. Clues to Determining Causes of Anxiety, Fear, and Panic Responses

Previous work in this laboratory largely focused on the neural substrates of anxiety, fear, and panic behaviors and physiology using animal models. It is well established in the clinic that while the majority of patients with PD will experience PAs to innocuous interoceptive stimulus, such as brief inhalations of 5-7% CO_2, healthy subjects display adaptive PAs to much higher (~35%) CO_2 concentrations [for review, see (Johnson et al., 2014)]. Similarly to the latter case, higher concentration of CO_2 (20%) elicits not only anxiety- and fear-associated behaviors in rodents, but also marked cardiovascular and thermoregulatory responses associated with panic states (Federici et al., 2019; Johnson et al., 2012c, 2011; Spiacci et al., 2018; Ziemann et al., 2009). Regarding PD, this laboratory developed a panic prone model of PeF GABA disinhibition in which panic prone rats, like humans with PD, also respond to innocuous 7% CO_2 with marked cardiovascular and thermoregulatory responses associated with panic states [(Fitz et al., 2003) for review, see (Johnson et al., 2014)]. Even though this dissertation will not use the GABA disinhibition panic prone model in rats, it is important to keep in mind that local 5-HT has been shown to hyperpolarize OX neurons in the PeFR (Chowdhury and Yamanaka, 2016; Muraki et al., 2004). In this respect, one should expect that antagonizing any neurochemical system that projects to and inhibits the PeFR may very well facilitate the precipitation of panic responses to innocuous stimuli, such as 7% CO_2.

Our laboratory has a long history of using telemetry probes implanted in the peritoneal cavity to measure the autonomic/physiological components of a panic-associated response to various anxiogenic or panicogenic stimuli. In addition to the commonly used

paradigms to assess important components of behavioral responses in anxiety, fear, and panic emotional states, in this dissertation I also assessed core autonomic/physiological features of panic utilizing telemetry probes to question the effects of 5-HT network manipulation in the cardiovascular parameters.

Chapter II: Materials and Methods for Chapters III and IV

2.1. Animals and Housing Conditions

All experiments were carried out in post-weening (45-55 g) or adult (300-350 g) male Wistar (Chapter III) or Sprague Dawley rats (Chapter IV) acquired from Envigo Laboratories (Indianapolis, IN, USA) and housed in plastic cages under standard environmental conditions (12/12 light/dark cycle with lights on at 7:00 AM, tested at light phase; and 22° C). Post-weening rats were used for virus injections and adult rats were used for serotonergic lesioning studies in the BLA or PeF. Food and water were provided *ad libitum*. Animal caretaking and experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Eighth Edition (National Research Council, 2011) and the Institutional Animal Care and Use Committee at Indiana University Purdue University at Indianapolis approved all procedures.

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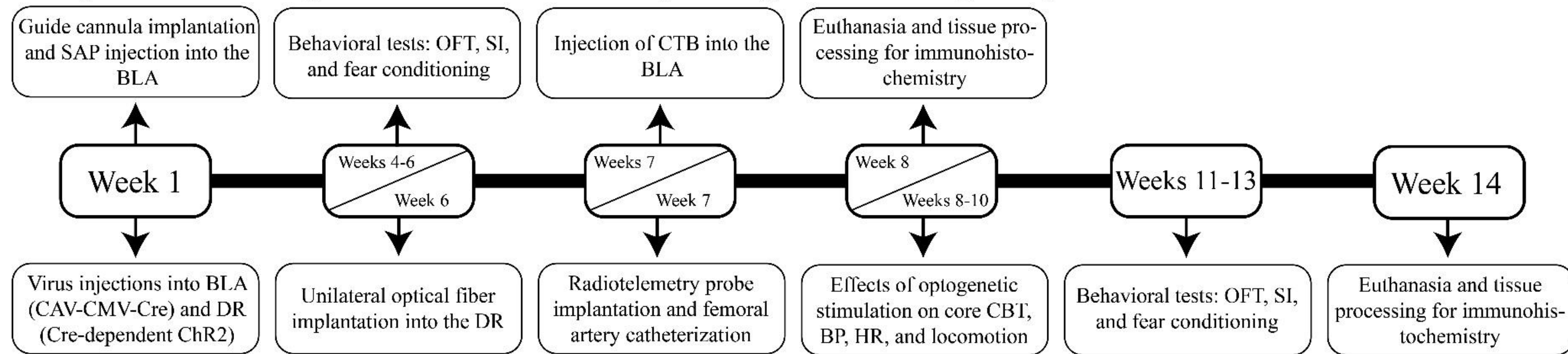
2.2. Experimental Timelines

Chapter 1 - Manipulation of BLA-Projecting Serotonergic System



Chapter 2 - Manipulation of PeF-Projecting Serotonergic System

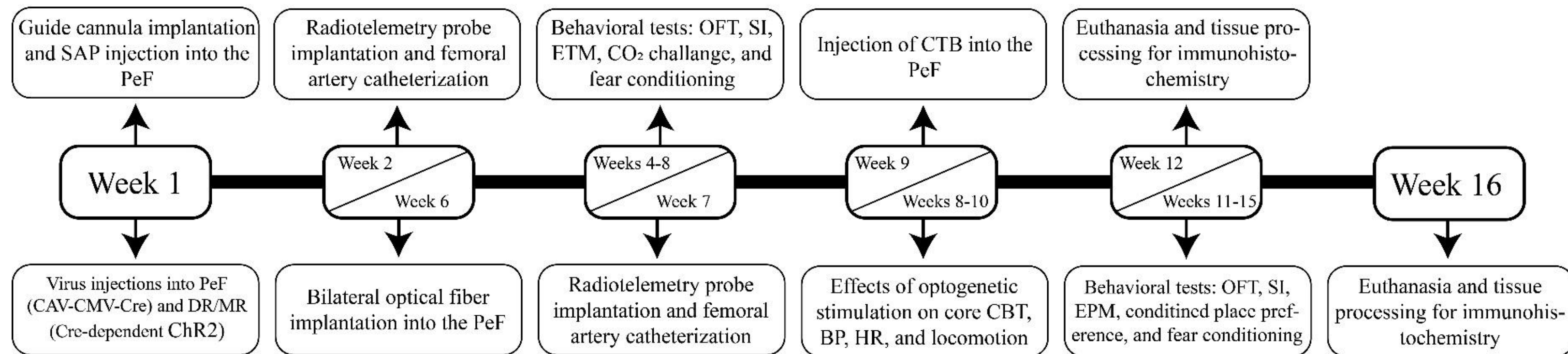


Figure 5 – Experimental timelines for Chapters III and IV. Abbreviations: BLA basolateral amygdala, BP blood pressure, CBT core body temperature, ChR2 channelrhodopsin, CO_2 carbon dioxide, CTB cholera toxin b subunit, DR dorsal Raphe nucleus, EPM elevated plus-maze, ETM elevated T-maze, HR heart rate, MR median Raphe nucleus, OFT open field test, PeF perifornical hypothalamus, SAP saporin toxin, and SI social interaction. For additional details on virus constructs, see section 2.3.1.

2.3. Surgical Procedures

All surgical procedures were conducted under sterile conditions. Rats were anesthetized under isoflurane delivered through a nose cone (2-3% by volume MGX Research Machine; Vetamac, Rossville, IN, USA, dissolved in pure oxygen; Praxair Inc., Indianapolis, IN, USA). Before commencing the surgery, rats were checked for corneal and paw withdrawal reflexes to ensure appropriate anesthesia. Animals' temperature were controlled with the aid of a rectal probe and a heating plate (TCAT-2DF; Physitemp, Clifton, NJ, USA). At the end of each surgical procedure, animals received three daily injections of carprofen (Levafen™ Injection, Patterson Veterinary, Greeley, CO, USA, 50 mg/ml, 5 mg/Kg dose, s.c.) for pain management.

2.3.1. Virus Injection and Optical Fiber Implantation Stereotaxic Surgeries

All viral injections were done in post-weening juvenile male Wistar (Chapter III) or Sprague Dawley rats (Chapter IV) that were group housed (2-3 animals) in plastic cages under standard conditions for 2-3 days prior to virus injection. Rats were placed on the stage of an ultraprecise stereotaxic instrument (David Kopf Instruments) using ear bars and with a nose cone connected to the isoflurane system (MGX Research Machine; Vetamac) during the virus injections.

Rats had their head cleaned with Betadine using Q-tips and with 70% alcohol. A midline incision on the animal's head was made with a surgical blade to expose the skull. The cranial aponeurosis was removed with a surgical blade, and the skull was dried and cleaned with 30% hydrogen peroxide to expose bregma and lambda. The incisor bar set so

bregma and lambda were on the same dorsoventral plane based on the tip of the glass pipette or injectors (33 gauge, C311I, Plastics One, Roanoke, VA, USA). Bregma was taken as reference for all subsequent virus injections and intracranial cannulations. All stereotaxic coordinates were according to a standard stereotaxic atlas of the adult rat brain (Paxinos and Watson, 1998).

Rats were first injected with 300 nl of the canine adenovirus CAV-CMV-Cre (4.3 x 10^{12} pp/ml, Institut de Génétique Moléculaire de Montpellier, France) bilaterally into the BLA [Chapter III, anteroposterior (AP): -1.48 mm; mediolateral (ML): ±4.25 mm; dorsoventral (DV):-8.00 mm] or PeF (Chapter IV, AP: -1.90 mm; ML: ±1.10 mm; DV:-7.20 mm) and then 300 nl of a Cre-dependent channelrhodopsin (ChR2) adeno-associated virus [4.4 x 10^{12} pp/ml, AAV-EF1a-DIO-ChR2-eYFP; University of North Carolina (UNC) Vector Core, Chapel Hill, NC, USA], or its control (5.1 x 10^{12} pp/ml, AAV-EF1a-DIO-eYFP; UNC Vector Core) unilaterally into the DR (Chapters III and IV, AP: -6.60 mm; ML: -1.40 mm; DV:-5.50 mm, 15 degree oblique to the midsagittal plane) with a flow rate set at 100 nl/min. The pipette was left in the site for 2 min and then removed slowly over 5 min to minimize backflow. These two-virus injections allowed us to specifically target DR/MR neurons projecting to either the BLA (Chapter III) or the PeF (Chapter IV), approach similar to those used by others (Junyent and Kremer, 2015; Schwarz et al., 2015). The head of the animals were sutured and they were allowed to recover from virus injections group-housed (2 to 3 per cage) in their home cages until they reached 300-350 g (approximately 5 weeks) and were ready for the wireless optical fiber implantation surgery.

Rats were connected to the isoflurane system (MGX Research Machine; Vetamac, Rossville, IN, USA) set at 2-3% (volume dissolved in pure oxygen; Praxair Inc., Indianapolis, IN) and secured to the ultraprecise stereotaxic frame. Their heads cleaned and skulls exposed for the unilateral or bilateral wireless optical fiber (TeleLC-B-6.0-500 Chapter III or TeleLC-B-8.9-500-2.5 Chapter IV, TeleOpto, Tokyo, Japan) implantation surgeries aiming the DRD/DRV (Chapter III, AP: -7.6 mm; ML: -1.2 mm; DV:-5.6 mm) or the PeF (Chapter IV, AP: -3.00 mm; ML: ±1.25 mm; DV:-7.70 mm), respectively. The wireless head implants were secured to the skull with the aid of four stainless steel screws (2.8 mm, Plastics One) anchored to the parietal and occipital bones, and the surgical site was filled with dental cement. Rats were individually housed thereafter and were allowed 7 days to recover at the housing facility and monitored daily for 5 days until the intraperitoneal radiotelemetry probe (HD-S11; Data Sciences International, New Brighton, MN, USA) implantation surgery and femoral artery catheterization.

2.3.2. Intracranial Cannulation and Specific Lesioning of the Basolateral Amygdala- or Perifornical Hypothalamus-Projecting Serotonergic Neurons with Saporin Toxin Conjugated to Anti-Serotonin Transporter

Guide-cannulas (26 gauge, C311G, Plastics One) were bilaterally implanted into the BLA (AP: -2.1 mm; ML: ±5.00 mm; DV:-8.5 mm, Chapter III) or the PeF (AP: -3.0 mm; ML: ±2.93 mm; DV:-8.5 mm, 15 degree oblique to the midsagittal plane, Chapter IV). The guide-cannulas were secured to the skull 1 mm above the injection site with the aid of 4 stainless steel screws (2.8 mm, Plastics One) on the parietal and occipital bones

and the surgical site was closed with dental cement. Each rat received two bilateral microinjections per site (100 nl each, 1 μM in artificial cerebrospinal fluid) of either saporin toxin (SAP) conjugated with SERT (SERT-SAP) or the control IgG-SAP (Kit-23, Advanced Targeting Systems, San Diego, CA, USA) via an injector (33 gauge, C311I, Plastics One) that fitted into and extended 1 mm over the guide-cannula (26 gauge, C311G, Plastics One). A 10 μl Hamilton syringe was placed on an infusion pump (PHD Ultra, Harvard Apparatus) and connected to the injectors with PE 50 tubing. Each microinjection lasted 5 min and the injectors remained in position for another 5 min and were slowly pulled out over 5 min. Upon completion of injections, dummy-cannulas (C311DC, Plastics One) that went the length of the guide cannula were screwed in place. Rats were individually housed thereafter and were allowed 7 days to recover at the housing facility and monitored daily for 5 days until the intraperitoneal radiotelemetry probe (HD-S11; Data Sciences International, New Brighton, MN, USA) implantation surgery and femoral artery catheterization. Behavioral assessments were carried out 3 weeks after intracranial injection of SAP. Volume, concentration, and timeline of SERT- and IgG-SAP injections were based on the previous work done by Nattie et al. (2004).

2.3.3. Tracing Studies: Cholera toxin B Subunit/Phaseolus vulgaris Leucoagglutinin Microinjections and Allen Brain Atlas Connectome.

To identify DR/MR projections to the BLA and PeF, an injector was inserted into the guide cannulas and 100 nl of the retrograde tracer cholera toxin b subunit (CTB, cat. no. 104, List Biological Laboratories, Campbell, CA, USA, 1% w/v in ACSF) was injected over 5 min into the BLA (Chapter III) or PeF (Chapter IV) three weeks prior to euthanasia.

A different cohort of Wistar rats received iontophoretic injection of Phaseolus vulgaris leucoagglutinin (Phal-l, cat. no. L-1110, Vector Laboratories, Burlingame, CA, USA, 2.5% w/v in ACSF) in the DR (AP: -6.5 to -7.5 mm; ML: 0.00 mm; DV:-5.5 mm) using positive current pulses of 10 µA (7 sec on; 7 sec off) for 15 min as a second method to confirm innervations originating from the DR.

To further confirm that there were serotonergic specific projections to the BLA (Chapter III), we utilized the connectome of the mouse brain in the Allen Brain Atlas (Oh et al., 2014) and found a SERT-Cre (Slc6a4-Cre_ET33) mouse (Gong et al., 2007) that was used in combination with local DR/MR injections of an AAV expressing a Cre-dependent fluorescent reporter to selectively identify serotonergic projections.

2.3.4. Radiotelemetry Probe Implantation and Femoral Artery Catheterization

The rats were submitted to the femoral artery catheterization to measure the cardiovascular (HR and BP) perturbations throughout different behavioral tests. The ventral skin was shaved and with the rat laying on its back, two incisions were made using a scalpel, one along the natural angle of the hind leg of approximately 1.5 cm exposing the femoral artery and one along the abdomen of approximately 2 cm exposing the peritoneal cavity. The DSI probes (HD-S11, Data Science International) were implanted in the peritoneal cavity and sutured to the internal wall of the abdominal muscle. With the aid of trocar, the pressure transducer catheter was tunneled into the hind leg incision and inserted into the femoral artery. The surgical sites were closed, and the animals were given 7 days of recovery time at the housing facility and monitored daily for 5 days until the beginning of the behavioral assessments.

2.4. Optogenetic Stimulation Parameters

The simulation pulses were generated by a software (Prizmatix Pulser, Version 2.3.1; Prizmatix, Southfield, MI, USA) connected to a programmable TTL pulse train generator (Prizmatix Pulser, Prizmatix) and a stimulator device (TeleOpto). The stimulation pulses were transmitted via infrared signals from an emitter (TELEEMITTER, TeleOpto) located above the apparatus where the animals were being stimulated. A wireless optogenetic receiver (TELER-3-P, TeleOpto) was connected to the optical fibers on the animal's head implant to receive the infrared signals from the emitter. The photostimulation parameters were dependent on the region (cell bodies versus fibers) and neuronal population being stimulated. The following stimulation parameters used for serotonergic cell bodies (470 nm, 20 Hz, 1 mW, 5 ms pulses, Chapter III), fibers (470 nm, 20 Hz, 10 mW, 5 ms pulses, Chapter IV), or glutamatergic cell bodies (470 nm, 10 Hz, 1 mW, 10 ms pulses, Chapter IV) were based on previous publications (Garcia-Garcia et al., 2017; Li et al., 2016; Marcinkiewcz et al., 2016; Molosh et al., 2018).

2.5. Behavioral Tests

All rats were handled for 5 min by the experimenter for two consecutive days prior to the first day of each experiment. Each behavior was video recorded with ANY-maze Video Tracking System (Version 5.33, Stoelting, Woods Dale, IL, USA) using a monochrome camera (DMK23UM021, The Imaging Source®, Charlotte, NC, USA). All experiments were conducted in a dark room with a red light (40 lux) and a background white noise (60 dB), unless specified otherwise.

2.5.1. Effects of Optogenetic Stimulation on Blood Pressure, Heart Rate, and Locomotion

Before the beginning of the experiments, all animals were habituated to the boxes where rats could move freely (50 cm width x 30 cm length x 40 cm height) for 20 min for 3 consecutive days. On experimental testing day, animals were placed in the experimental box and once a baseline was established for BP, HR, and locomotion, rats were optogenetically stimulated (Chapters III and IV) for 5 min. Animals' behavior and physiological responses were recorded for up to two hours after optogenetic stimulation.

2.5.2. Open Field Test

The open field test (OFT) covered a squared area of 90 cm x 90 cm, with 40 cm height walls which was divided into a 6 x 6 grid of equally- sized squares using black tape (36 total squares) with 4 squares forming the center; 12 squares forming the middle perimeter; and 20 squares forming the outer perimeter. On the test day, rats were placed in the center of the apparatus and the time spent in the different zones during the 5 min test was video recorded and scored using ANY-maze (Version 5.33, Stoelting) video tracking software (Stoelting, Woods Dale, IL, USA). Whenever applicable, animals were optogenetically stimulated during (Chapters III and IV) the OFT.

2.5.3. Social Interaction Test

The SI test is an experimental anxiety-like behavior test in rats (File, 1980), and was conducted as described previously (Sanders et al., 1995). The 'experimental' rat and an unfamiliar 'partner' rat were placed individually in the center of the open field 24 hours before the SI test. During the SI test, the two rats were placed on opposite corners of the

open field, and the total duration (seconds) of non-aggressive physical contact (grooming, sniffing, and crawling over and under) initiated by the 'experimental' rat was video recorded and manually scored by a blind experimenter. Whenever applicable, rats were submitted to optogenetic stimulation for the duration of the SI test.

In Chapter III, animals were submitted to one trial of SI test. In Chapter IV, animals were submitted to four different SI tests with novel partners in each session, namely: baseline, stimulation, bright light challenge, and CO_2 challenge. The bright light challenge consisted of an abrupt transition from dim red light (40-watt red light, 1 lux) to bright white fluorescent lighting (488 lux) during the 5-min SI testing session (File and Hyde, 1978). The CO_2 challenge consisted of pre-exposure of the "experimental" rat to a Plexiglas® gas chamber (50 cm width x 30 cm length x 40 cm height) with 20% CO_2 (normoxic conditions, Praxair Inc., Indianapolis, IN) for 5 min prior to SI test (Johnson et al., 2005). The animals were optogenetically stimulated for the duration of the SI test for all sessions except baseline, and the total duration (seconds) of non-aggressive physical contact (grooming, sniffing, crawling over and under and so on) initiated by the 'experimental' rat was manually score by a blind experimenter.

2.5.4. Fear Conditioning

To investigate how the loss (lesioning with SERT-SAP) or gain of function (intersectional genetics) manipulations of the serotonergic neurotransmission to the BLA (Chapter III) or PeF (Chapter IV) would alter the expression of the freezing behavior to the conditioned fear, rats were exposed to the fear conditioning paradigm. The fear conditioning protocol was 4 days long (Johnson et al., 2015b) and was implemented 3 or 6 weeks after

stereotaxic surgeries to allow lesioning of SERT terminals or expression of the viral constructs to take place, respectively. All rats were handled for 5 min by the experimenter prior to day 1 of the fear conditioning experiment. For the fear conditioning experiments in the gain of function approach, rats were optogenetically stimulated in their home cages for 5 min prior to acquisition, consolidation, and recall/extinction sessions.

On day 1 – habituation session – rats were exposed for 10 min to the conditioning box (25.5 cm length x 25.5 cm width x 39.5 cm height) with a grid floor that was connected to a scrambled shock generator (Ugo Basile, Monvale, Italy). The conditioning box was placed in a larger sound-attenuated chamber (Ugo Basile) with a speaker in the rear wall of the chamber to provide a 60 dB background noise and a white 15-lux light on the ceiling to illuminate the chamber during all sessions.

On day 2 – acquisition session – rats were submitted to 5 trials consisting of a 20 s, 4 kHz, 80 dB tone (CS) that co-terminated with a 0.5 s, 0.8 mA single footshock (US) with inter-trial interval (ITI) of 100 s.

On days 3 and 4 – consolidation and recall/extinction sessions – rats were exposed to 5 (consolidation) or 20 (extinction) CS only, respectively.

Total time freezing during the CS presentations (20s tone) was recorded and manually scored by a blind experimenter for each rat. Freezing was defined as the absence of all movement except for normal breathing. Before each trial, the chamber and the conditioning box were cleaned with 70% ethanol to minimize any olfactory cues.

2.5.5. Real-Time Place Preference/Avoidance Test

To test whether stimulating the glutamatergic cell bodies in the PeF or the serotonergic terminals of the DR/MnR-PeF projections (Chapter IV) would support place preference or avoidance, we placed rats into a two-chamber apparatus in the real-time place preference/avoidance (RTPP/A) test where one side of the chamber was paired with optical stimulation (modified from Nieh et al., 2016). The RTPP/A apparatus was made of two equal sized boxes (30 cm width x 45 cm length x 30 cm height), divided by a center wall and connected by a 13 cm diameter opening. Each box presented different visual cues covered in black and white checkered or stripped paper on the walls and floor. The behavior was performed over three days in four different sessions lasting 20 min each. During habituation session on day one, each animal was assigned to either the stripped or checkered box and allowed to explore the apparatus. The box that the animal was first placed during the habituation session on day one became the stimulation-off box whereas the opposite box became the stimulation-on box for all the subsequent sessions of the test and rats were assigned to each side in a counterbalanced manner. During the stimulation session on day two, animals were placed in the stimulation-off box and allowed to explore the apparatus and were photostimulated whenever they moved into the stimulation-on box. Forty minutes and 24 hours after the stimulation session on day 2 (respectively short- and long-term sessions hereafter) the animals were placed back in the stimulation-off box and tested without any optogenetic stimulation.

Each session was video recorded and manually scored later by a blind experimenter for the duration each animal spent in each box. The animal was defined to be inside each

area when at least 50% of its body was completely located inside the designated compartment. We utilized the difference score (percentage time spent in the stimulation-on box minus percentage time spent in the stimulation-off box) to determine place preference/avoidance.

2.5.6. Elevated Plus-Maze Test

To test whether stimulating the PeF-projecting serotonergic terminals (Chapter IV) would overcome the aversive properties of the open arm in the EPM, we placed rats into the central platform of the EPM and paired the time spent in the open arm with photostimulation. The EPM (Pellow and File, 1986) consisted of two open arms (50 cm length x 10 cm width x 1cm height rim to prevent falls), and two closed cm arms (50 cm length x 10 cm width x 40 cm height) with high walls and open roof such that the two arms of each type were opposite to each other (**Figure 6**). The apparatus was elevated to a height of 50 cm from the floor. The animals were placed on the central platform facing the open arm and were allowed to explore the maze for 10 min. Rats were paired with photostimulation whenever 50% of their body was in the open arm during the first 5 min. Animals were allowed to explore the apparatus without optogenetic stimulation for the last 5 min of the test. The time spent in each arm was scored using ANY-maze (Version 5.33, Stoelting).

2.5.7. Elevated T-Maze Test

To determine whether lesioning the serotonergic projections from to the PeF would facilitate the escape responses from the open arm (Chapter IV), rats were tested in the

ETM. The ETM is a modification of the EPM in which one of the closed arms is blocked, with

Figure 6 – Photo illustrating the elevated plus maze.

the animal having access only to two open arms and one closed arm in a “T” shape. For two consecutive days before the pre-exposure session, animals were handled by the experimenter for 5 min for habituation purposes. During the pre-exposure session, animals were exposed to one of the open arms of the ETM for 30 min. A barrier was place at the proximal part of the open arm, right before the intersection with the closed arm to prevent the animals from accessing the rest of the maze and limiting their exploration only to the open arm. The barrier was removed after the pre-exposure session was finalized. On the test day, the animals were exposed 3 times to the distal ends of the closed and open arms with ITI fixed at 30 seconds, with a total of 6 trials. The time taken by the animal to leave the closed and

open arms were determined as inhibitory avoidance and escape behaviors, respectively. A cutoff value of 300 seconds was established before terminating the trial in case the animals failed to complete the task (Zangrossi and Graeff, 1997). All behaviors were video recorded and later scored manually by an experimenter blind to treatments. Immediately after the last exposure to the open arm, the animals were placed in the open field to test the baseline locomotor activity.

2.5.8. CO_2 Challenge

Since lower subthreshold concentrations of hypercapnic gas (e.g. 5-7% CO_2) elicit maladaptive PAs (increases in fear/anxiety and cardiorespiratory responses) in the majority of patients with panic disorder, but not in healthy controls (Gorman et al., 1984; Klein, 1993), this model was also used in animals to measure the role of 5-HT modulation on escape responses elicited by low (7.5%) and high (20%) concentrations of CO_2 [(Johnson et al., 2011) for review, see (Johnson et al., 2014)].

Flow cages (50 cm width x 30 cm length x 40 cm height) were custom built using Plexiglas®. When the lid of the cage was latched, gases could only enter the cage through an inlet connector (for the gas infusion) and could only exit the cage through an outlet connector. The gas flow into the cages was controlled using a two-stage regulator (Praxair, Inc.) at a pressure of 0.6 Bar. Rats were habituated for 10 min to the flow cages prior to CO_2 challenge, which was carried out in two different concentrations: 7.5 and 20% in a counterbalanced manner. Also, a crossover design was utilized so that all rats received either 7.5 or 20% CO_2 within one week between each challenge.

All rats (Chapter IV) were submitted to constant infusion of atmospheric air (< 1% CO_2 / 21% O_2 / 79% N_2, Praxair, Inc.) until they ceased exploratory behavior and there was a 5 min stable baseline for the cardiovascular parameters (i.e., ~110 mm/Hg and ~400 bpm). Then a premixed experimental normoxic, 7.5 or 20% hypercapnic gas (7.5% or 20% CO_2, 21% O_2, 71.5% or 59% N_2: Praxair, Inc.) was infused for 5 min to increase the CO_2 concentration in the flow cage to the pre-established values (**Figure 7**). The CO_2 and O_2 gas concentrations were measured in real time by UV sensors (CM-0121 and CM-0201, respectively, CO2meter.com, Ormond Beach, FL, USA) connected to the flow cage outlet and to a software (Gaslab v2.0.8.14, CO2meter.com). Whenever applicable, rats were also optogenetically stimulated for 5 min during the CO_2 infusion. Atmospheric air infusion resumed right after termination of CO_2 infusions and remained for additional 40 min. The CO_2 levels rapidly returned from 7.5 or 16% to < 3% during the following 7 min. Note that the concentration of O_2 remained constant at ~21% throughout the entire gas (either CO_2 or atmospheric air) infusion. (**Figure 7**).

2.6. Immunohistochemistry

In the end of experiments, rats were anesthetized with isoflurane and transcardially perfused with 200 ml of 0.1 M phosphate buffered saline (PBS) and then 200 ml 0.1 M PBS containing 4% paraformaldehyde. Brains were removed and postfixed with 0.1 M PBS containing 4% paraformaldehyde for 3 hours before transferring to anti-freezing solution [0.1 M phosphate buffer (PB) containing 30% sucrose] for 48–72 h until they sank and were ready for sectioning. Brains were sectioned (30 µm serial sections) using a microtome (model HM 400 R, Micron, Walldorf, Germany) with a cooling system (model K

4000, Micron) and immediately placed in cryoprotectant solution (30% ethylene glycol and 30% glycerol in 0.1 M PB). Slices were stored at -20 ºC until the beginning of free-floating immunohistochemistry as described previously (Johnson et al., 2005).

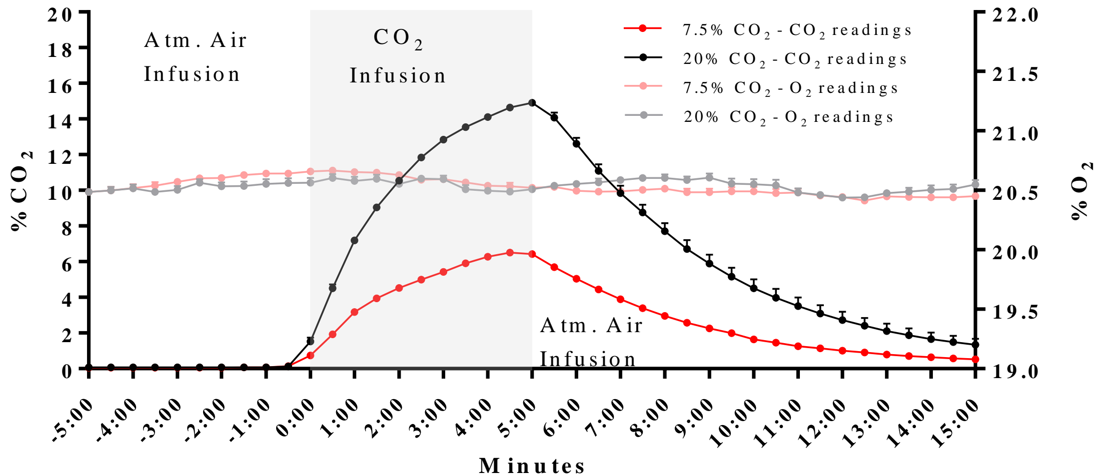


Figure 7 – Graph showing percent concentration of gases during the carbon dioxide (CO_2) challenge. Real-time CO_2 (left y-axis) and oxygen (O_2, right y-axis) readings over 20 min (x-axis) infusion of atmospheric (Atm.) air, 7.5, and 20% CO_2 gas infusion in the gas chamber. Readings were taken during all CO_2 challenge experiments in Chapter IV and no significant difference between groups were found for gas concentration over the course of the experiments.

Our general chromogen immunohistochemistry procedure was as follows: sections were incubated on day 1 for 30 min in PBS, 20 min in 1% H_2O_2 in PBS, 30 min in PBS, 10 min in PBS with 0.3% Triton X-100 (PBST), and overnight (approximately 12–16 h) in primary antibody diluted in PBST (for list of antibodies used, see **Table 3**). On day 2, sections underwent 30 min in PBS, 90 min incubation with the appropriate secondary antibody diluted in PBST. Following the secondary antibody incubations, slices were washed in PBST for 30 min, incubated 90 min in avidin-biotin complex kit (cat no. PK-6100, Vector Laboratories) diluted 1:500 in PBST, 30 min washes in BPST, and 5-10 min chromogen reaction with SG Kit (SK-4700, Vector Laboratories) for cFos, CTB, and SERT primary

antibodies or 0.01% 3,3'-diaminobenzidine tetrahydrochloride (DAB; cat no. D-5637, Sigma-Aldrich, Poole, UK) in PBS containing 0.003% H_2O_2, pH 7.4 for Phal-l and tryptophan hydroxylase (TPH) primary antibodies. The slices submitted to double staining underwent an additional overnight incubation with the appropriate primary antibody. On day 3, the protocol was identical to the steps carried out on day 2, but it was followed by 7 min DAB reaction. After finishing the immunohistochemistry, brain slices submitted to cFos or Phal-l immunoreactions were also counter stained with neutral red or cresyl violet, respectively, to facilitate identification of brain nuclei and cell counting.

Our general procedure for immunofluorescence was as follows: sections were incubated for 30 min in PBS, 10 min in PBST, and overnight in primary antibody (for list of antibodies used, see **Table 3**). On day 2, sections underwent 30 min in PBS, 90 min incubation with the appropriate secondary antibody diluted 1:200 in PBS. Following the secondary antibody incubations, slices were washed in PBS for 30 min and those submitted to double labelling underwent an additional overnight incubation with the appropriate primary antibody. On day 3 the protocol was identical to the steps carried out on day 2, except the secondary antibody. All washes and incubations were done in 12 well polystyrene plates with low frequency shaking on an orbital shaker.

All sections were mounted in 0.1% gelatin dissolved in ultrapure water on glass slides, dried overnight, and mounted with coverslips using VectaShield mounting medium (cat. no. H-1200, Vector Laboratories, for immunofluorescence) or dried overnight, dehydrated and mounted with coverslips using DPX mounting medium (BDH Laboratory Supplies, Poole, UK, for brightfield immunohistochemistry).

Table 3 – Summary of staining combinations and antibodies used.

Type of reaction	Neurochemical markers	Primary antibodies	Secondary antibodies
Chromogen	Phal-l	Goat anti-Phal-l (1:1000)	Rabbit anti-goat (1:200)
	cFos	Rabbit anti-cFos (1:500)	Goat anti-rabbit (1:200)
	SERT	Rabbit anti-SERT (1:1000)	Goat anti-rabbit (1:200)
	cFos + TPH	Rabbit anti-cFos (1:500) Mouse anti-TPH (1:1000)	Goat anti-rabbit (1:200) Horse anti-mouse (1:200)
	cFos + OXA	Rabbit anti-cFos (1:500) Rabbit anti-OXA (1:12000)	Goat anti-rabbit (1:200) Goat anti-rabbit (1:200)
	TPH + CTB	Mouse anti-TPH (1:1000) Goat anti-CTB (1:1000)	Horse anti-mouse (1:200) Rabbit anti-goat (1:200)
Fluorescence	TPH	Mouse anti-TPH (1:300)	Horse anti-mouse (1:200)
	TPH + eYFP	Mouse anti-TPH (1:300) N/A	Horse anti-mouse (1:200) N/A
	TPH + CTB	Mouse anti-TPH (1:300) Goat anti-CTB (1:1000)	Horse anti-mouse (1:200) Rabbit anti-goat (1:200)
	OXA + eYFP	rabbit anti-OXA (1:2000) N/A	Rabbit anti-goat (1:200) N/A
	SERT + eYFP	Rabbit anti-SERT (1:500) N/A	Goat anti-rabbit (1:200) N/A

List of antibodies and vendors: goat anti-CTB (cat. no. 703, List Biological Laboratories, Campbell, CA, USA); goat anti-Phal-l antiserum (cat. no. AS-2224, Vector Laboratories, Burlingame, CA, USA); mouse anti-TPH antibody (cat. no. T0678, Sigma, St. Louis, MO, USA); rabbit anti-cFos (cat. no. SC52, Santa Cruz, Dallas, TX, USA); rabbit anti-orexin A (OXA, H003-30, Phoenix Pharmaceuticals, Burlingame, CA, USA); rabbit anti-SERT (cat. no. 24330, ImmunoStar, Hudson, WI, USA); biotinylated goat anti-rabbit, horse anti-

mouse, rabbit anti-goat (cat nos. BA-1000, BA-2000, BA-5000, respectively, Vector Laboratories); biotinylated donkey anti-goat (cat. no. 705-066-147, Jackson Immunoresearch, West Grove, PA, USA); donkey anti-goat Alexa Fluor 568, goat anti-mouse Alexa Fluor 488 (cat. nos. A-11057, A-11017, respectively, Life Technologies, Carlsbad, CA, USA); and donkey Cy™3-conjugated anti-rabbit (cat. no. 711-165-152, Jackson ImmunoResearch, West Grove, PA, USA).

2.7. Photography and Image Analyses

Photomicrographs were obtained with a microscope (DMLB, Leica, Wetzlar, Germany) connected to a digital camera (DFC 300, Leica) or a confocal scanning microscope (A1R+, Nikon, Tokyo, Japan). Densitometry analyses of SERT$^+$ fibers were done on grayscale inverted photographs from the BLA (-2.8 mm from bregma) and PeF (-3.0 mm from bregma) using an image editing program (Photoshop version 16, Adobe Systems, San Jose, CA, USA). The IgG-SAP group mean represented 100%, which was compared to SERT-SAP values. The numbers of cFos$^+$ cells were counted in the entire field of view at 400x magnification in the BLA, PeF, MR, and subdivisions of the DR (-7.8 mm from bregma). The co-localization analysis was determined using Pearson's correlation coefficient with Nikon software (Nikon NIS Elements, Nikon). An observer that was blind to the experimental treatment of each animal did all cell counts.

2.8. Statistical Analysis

The following dependent variables were analyzed using a two-tailed independent Student's t-test: densitometry, social interaction, total freezing duration, and cell counts). The co-localization analysis of SERT and ChR2-eYFP was determined using Pearson's correlation coefficient with Nikon NIS Elements software. Conditioned fear responses (i.e. freezing) were analyzed using a one-way analysis of variance (ANOVA) or a one-way

ANOVA with repeated measures with SAP or AAV injections as between-subjects factors and time as repeated measures. In the presence of significant main effects, between-subjects post hoc tests were conducted using Fisher's least significant difference (LSD) test. Within subjects time effects were also assessed using a Dunnett's one way analysis with the min prior to stimulation (baseline) used as the control. Statistical significance was accepted with $p \leq 0.05$. All statistical analyses were carried out using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and all graphs were generated using GraphPad Prism 7.04 for Windows (GraphPad Software Inc. La Jolla, CA, USA) and figure plate illustrations were done using CorelDraw version X8 for Windows.

Chapter III: Using loss and gain of function approaches to manipulate amygdala-projecting serotonergic neurons in the dorsal Raphe nucleus that enhance anxiety-related and conditioned fear behaviors

3.1. Introduction of Chapter III

Selective serotonin reuptake inhibitors (SSRIs) are the first line of pharmacological treatment for anxiety disorders, such as generalized anxiety disorder and social anxiety disorder, as well as trauma- and stressor-related disorders, such as posttraumatic stress disorder (PTSD) [for review, see (Koen and Stein, 2011)]. The anxiolytic effects of SSRI therapy occur 2-3 weeks following daily treatments and there is evidence that they actually increase anxiety initially (Masand and Gupta, 1999; Spigset, 1999; Teicher et al., 1990). For instance, in humans, taking citalopram prior to fear acquisition increases fear-potentiated startle response (Browning et al., 2007; Grillon et al., 2007), but chronically reduces conditioned fear response (Bui et al., 2013). Similarly, in rodents SSRIs initially enhance conditioned fear behavior, but chronically will reduce conditioned fear response [(Burghardt et al., 2007, 2004) for review, see (Burghardt and Bauer, 2013)]. Thus, the mechanism of action appears to be compensatory changes that occur within anxiety and fear networks with repeated use. In light of this, we and others hypothesize that a hyperactive or hyperresponsive central serotonergic system contributes to increases in anxiety sensitivity in anxiety disorders and to exacerbated anxiety/fear in response to SSRIs acutely, but over time becomes desensitized with chronic SSRIs.

A critical part of the innate and learned fear network is the basolateral amygdala (BLA; which includes the basolateral and lateral nuclei). The BLA is highly responsive to

various stress-inducing stimuli (Brydges et al., 2013; Butler et al., 2011; Henderson et al., 2012; Johnson et al., 2008; Singewald et al., 2003) and increase in amygdala activity is associated with increase in anxiety in humans (Rauch et al., 2003). The amygdala plays a critical role in fear conditioning in rodents [for reviews, see (Johansen et al., 2012, 2011)], and in humans. For instance, humans with bilateral ablation of the amygdala: 1) have normal facial recognition but do not recognize fearful faces (Adolphs et al., 1994); 2) do not display fear in response to normally threatening stimuli (Feinstein et al., 2011); and 3) do not show conditioned fear response, even though declarative learning is intact (Bechara et al., 1995).

Serotonergic neurons are clustered within the midbrain in the dorsal (DR) and median Raphe (MR) nuclei and within the medulla, but the vast majority of serotonergic neurons are localized in the DR. Serotonergic fibers are dense in the BLA and primarily originate from the midline ventral (DRV) and dorsal (DRD) DR, with far fewer projections from the MR (Hale et al., 2008). Extracellular levels of 5-HT increase in the BLA during conditioned fear (Zanoveli et al., 2009) and in response to inescapable stress (Amat et al., 1998). Meanwhile, administration of anxiogenic drugs with diverse pharmacological properties, or administering a light cue that was previously paired with a shock increases cFos in 5-HT neurons in the BLA-projecting regions of the DRV and DRD (Abrams et al., 2005; Spannuth et al., 2011). Additionally, single systemic injection of an SSRI in rats increases extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001), enhances freezing responses during acquisition and consolidation [(Ravinder et al., 2013) for review, see (Burghardt and Bauer, 2013)]. Up to 24 h following exposure to inescapable stress, baseline concentrations of 5-HT in the BLA are increased, and animals show increased response

to two brief footshocks, suggesting that inescapable stress can sensitize BLA-projecting serotonergic system (Amat et al., 1998).

The persistent increase in extracellular 5-HT concentration within the amygdala following stress may contribute to a net loss of local GABA inhibition and subsequent increase in excitation of glutamatergic projection neurons. In support of this, 5-HT acutely increases GABAergic tone in the BLA by exciting local GABAergic interneurons via the postsynaptic 5-HT_{2A} receptors (Jiang et al., 2009; McDonald and Mascagni, 2007; Rainnie, 1999), but also facilitates synaptic plasticity of pyramidal neurons via NMDA-dependent mechanisms upon activation of 5-HT_{2C} receptors (Chen et al., 2003). Equally important is that stress can downregulate the 5-HT_{2A} receptor and reduce 5HT's effects on local GABAergic tone (Jiang et al., 2009), but also enhance cell surface expression of 5-HT_{2C} receptors in the amygdala (Baratta et al., 2016), which may bias toward excitation of 5-HT_{2C} receptors on fear-promoting BLA glutamatergic projection neurons. Overall, these studies suggest that serotonergic projections to the BLA play a role in modulation of anxiety-like behavioral responses and conditioned fear behavior.

Yet, these data are correlational and pharmacological methods of understanding the role of 5-HT in fear are complicated in that 5-HT is capable of exciting and inhibiting both GABA and glutamatergic neurons in BLA. In order to test our hypothesis that 5-HT projections from the DRD/DRV region of the DR promote anxiety- and fear-related behaviors, in recent studies we injected a serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the BLA, which reduced local 5-HT concentration (but not dopamine, norepinephrine or epinephrine) and attenuated anxiety-like behavioral response in the social interaction test and cue-induced conditioned fear response (Johnson et al., 2015b). These

data supported our hypothesis, but the mechanism of action of 5,7-DHT is largely unknown and requires co-injection of the norepinephrine reuptake inhibitor, desipramine, in order to be selective. In order to further explore our loss-of-function approach, we first injected a selective anti-serotonin transporter (SERT)-saporin (SAP) toxin into the BLA (**Figure 8**) and assessed anxiety-like and conditioned fear responses, then injected a retrograde tracer into BLA to confirm that we not only lesioned SERT-immunoreactive fibers in the BLA but also serotonergic neurons in the DRD/DRV. We then utilized optogenetic approach to excite BLA projecting 5-HT neurons in the DR (**Figure 8**) while assessing anxiety-like and conditioned fear behaviors.

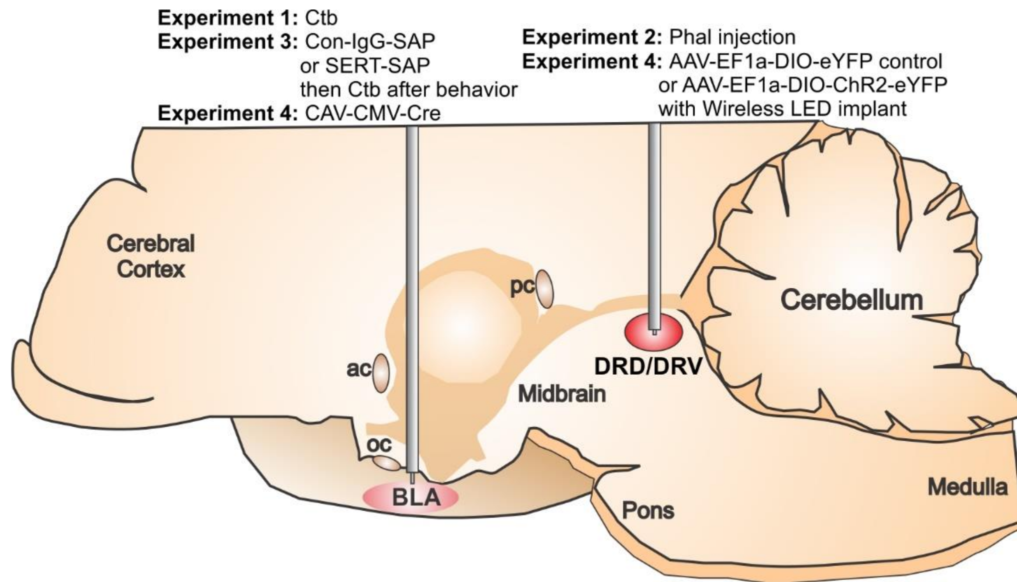


Figure 8 – Illustration of a midsagittal section of a rat brain indicating the intracranial injections carried out in Chapter III. Illustration of what tracers [i.e., Phaseolus vulgaris leucoagglutinin (Phal) or Cholera Toxin B (CTB)], toxins [i.e., control IgG conjugated to saporin (Con-IgG-SAP) or anti-serotonin transporter conjugated to saporin (SERT-SAP)], or viruses [i.e., CAV-CMV-Cre, AAV-EF1a-DIO-eYFP control, or AAV-EF1a-DIO-ChR2-eYFP] were injected into the dorsal and ventral dorsal Raphe nucleus (DRD/DRV) or basolateral amygdala (BLA) from each experiment. Abbreviations: ac, anterior commissure, pc, posterior commissure, oc, optic chiasm.

3.2. Results

3.2.1. ***Experiments 1 and 2:*** *Effects of retrograde and anterograde tracing of potential DR projections to BLA*

Our experiment 1 showed that injections of the retrograde tracer CTB into the BLA (**Figure 8** and **Figure 9a**) led to CTB expression in many TPH^+ neurons in the DRD and DRV (**Figure 9c-d**), but few in the lwDR or MR (**Figure 9e-f**, see topography of midbrain TPH^+ neurons in **Figure 9b**, n=4) with only 5-8% of CTB being expressed in TPH- neurons.

Anterograde Phal injections into the midbrain midline DR in the experiment 2 (**Figure 8**) induced expression of Phal within the DR that was most highly expressed in the DRV, then DRD (**Figure 10a**). $Phal^+$ fibers were most highly expressed in the BLA, but also in surrounding cortex and CeA (**Figure 10b-c**). In experiment 480074702-DR from the Allen Brain Atlas (mouse connectivity), injecting a Cre-dependent AAV-hSyn-EGFP construct into the midline DR of a SERT-Cre mouse led to local expression of EGFP fluorescence in the DR (**Figure 10d**), distal EGFP fluorescence in serotonergic fibers in the BLA and CeA and surrounding cortex (**Figure 10e**).

3.2.2. ***Experiment 3:*** *Effects of SERT-SAP injections in the BLA on behavior and immunohistochemical validation of approach*

Compared to the control IgG-SAP group, injection of SERT-SAP into the BLA significantly reduced the density of local SERT+ fibers in the BLA by ~90% ($t(9)=19.6$,

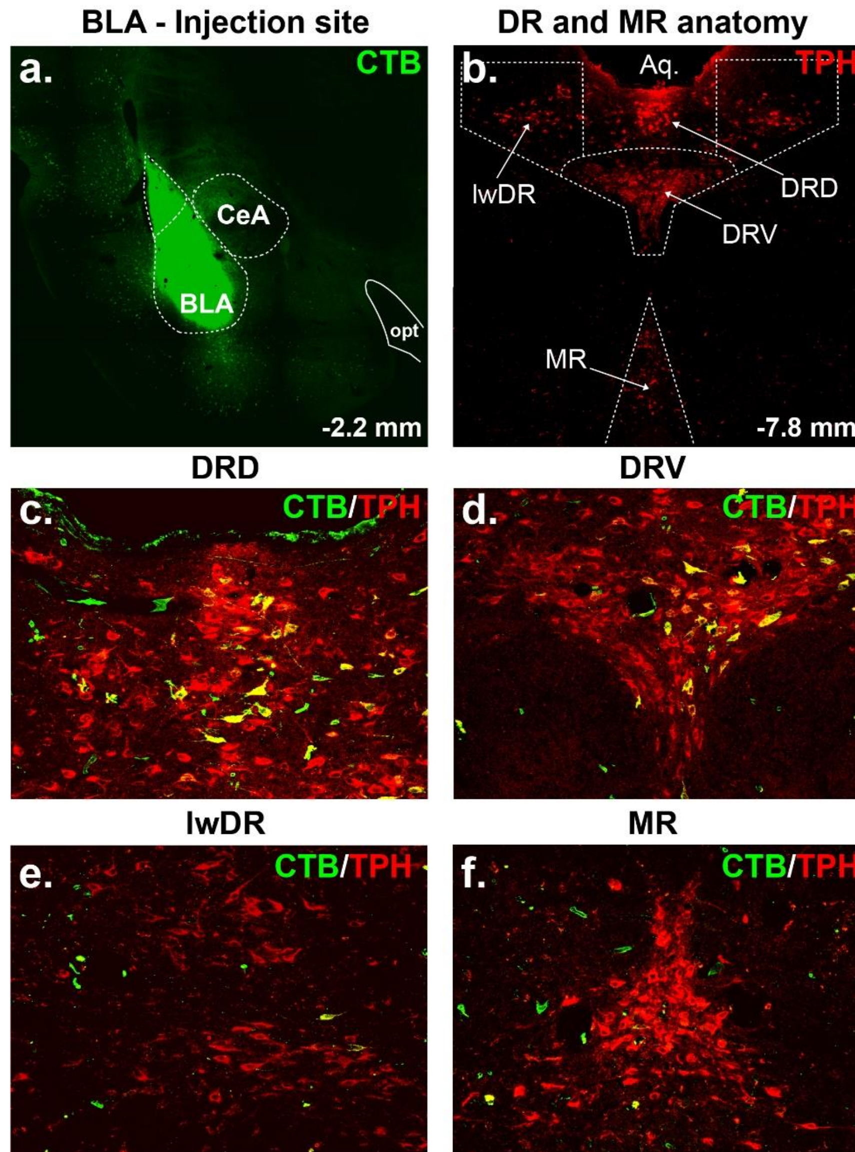


Figure 9 – Retrograde tracing from the basolateral amygdala (BLA). **(a)** Injection site illustrating Cholera Toxin b subunit (CTB, Alexa 488, green) injection into the BLA. **(b)** Topography of tryptophan hydroxylase (TPH, Cy3, red) immunoreactive serotonergic neurons in the midbrain Raphe nuclei [i.e., dorsal (DRD), ventral (DRV) and lateral wing (lwDR) divisions of the dorsal (DR) and median Raphe (MR) nuclei] In panels **(c)** through **(e)** are immunohistochemistry reactions against TPH and CTB highlighting many BLA projecting neurons originating from **(c)** DRD and **(d)** DRV, but only a few from **(e)** lwDR and **(f)** MR. Abbreviations: CeA, central amygdala; opt, optic tract.

$p<0.001$; Figure 11a, n=6 IgG-SAP, 5 SERT-SAP, one section damaged in control IgG-SAP group). In contrast, injection of SERT-SAP into the BLA resulted in approximately 10% reduction in the density of SERT+ fibers in the perifornical hypothalamus (control

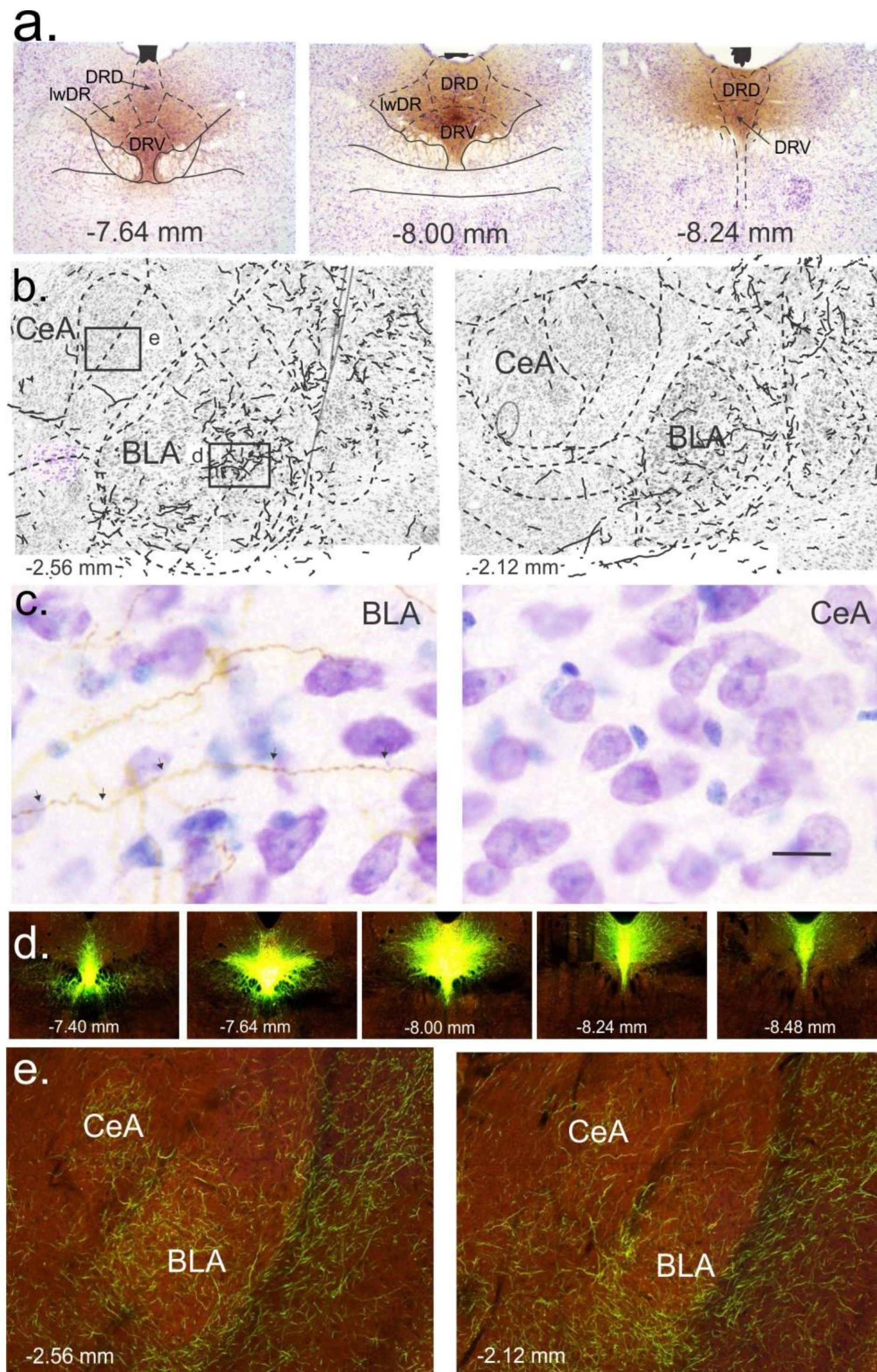


Figure 10 – Anterograde tracing from the rat and mouse dorsal Raphe nucleus to the basolateral (BLA) and central (CeA) amygdala. **(a)** Coronal sections of the midbrain and pontine dorsal Raphe nucleus (DR) illustrating *Phaseolus vulgaris* leucoagglutinin (Phal)-immunostained cells and fibers following injection of the anterograde tracer into the midline dorsal (DRD) and ventral (DRV), but not lateral wings (lwDR) divisions of the DR

[see orange/brown 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen immunostaining of Phal with purple cresyl violet staining of cells]. Numbers at bottom of each photograph indicate rostrocaudal level [mm from bregma; (Paxinos and Watson, 1998)]. **(b)** Coronal sections of the BLA and CeA nuclei of the amygdala with dashed line delineating nuclei and solid lines drawn over Phal-immunoreactive anterograde projections from DRD/DRV at two bregma levels indicated at bottom left. Boxes in the illustration on the left indicate where high magnification photos were taken and shown in **(c)** for BLA and CeA (orange/brown Phal-immunoreactive fibers with purple cresyl violet-stained cells. **(d)** Photos represent coronal sections of the DR from a serotonin transporter (SERT)-Cre mouse where a Cre-dependent AAV with a fluorescent reporter was injected into the DR and provided by the connectome of the Brain in the Allen Brain Atlas (Oh et al., 2014). **e)** Photos are coronal sections of the BLA and CeA from the SERT-Cre mice in **(d)** showing expression of 5-HT terminals from the midline DR. Image credit for parts **(d)** and **(e)**: Allen Institute for Brain Science.

site, data not shown), which receives sparse collateral projections from 5-HT neurons that project to the BLA (Muzerelle et al., 2016). Intra-BLA injections of SERT-SAP had no effect (i.e., no treatment x time interaction or main effect of treatment) on fear acquisition ($F(1,10)=0.3$, $p=0.591$; $F(1,10)=0.4$, $p=0.550$, Figure 11b, n=7 IgG-SAP, 5 SERT-SAP) and consolidation ($F_{(4,40)}=1.2$, $p=0.305$; $F_{(1,10)}=2.3$, $p=0.161$, not shown), but did reduce overall freezing response during fear recall/extinction [i.e., there was a main effect of treatment (line graph $F_{(1,10)}=7.52$, $p=0.02$, bar graph $t_{(10)}=2.5$, $p=0.03$), **Figure 11c**, n=7 IgG-SAP, 5 SERT-SAP, but no treatment x time interaction ($F_{(4,40)}=0.6$, $p=0.645$)]. No effects of intra-BLA injection of SERT-SAP on SI were detected ($t_{(10)}=1.1$, $p=0.285$, **Figure 11d**, n=7 IgG-SAP, 5 SERT-SAP). In control IgG-SAP rats, bilateral injections of CTB into the BLA led to CTB expression in many TPH^+ neurons in the DRD and DRV, but few in the lwDR or MR (see topography of midbrain TPH^+ neurons in **Figure 9a**, and co-localization between CTB and TPH in the DRD, DRV, lwDR, and MR in **Figure 11e**) with 5-8% of CTB being expressed in TPH- neurons. SERT-SAP injections into the BLA also reduced the total number of TPH^+ neurons in the DRD ($t_{(10)}=2.1$, $p<0.05$) and DRV ($t_{(10)}=1.7$, $p<0.05$), but not in the lwDR ($t_{(10)}=0.98$, $p=0.349$) or MR ($t_{(10)}=1.34$, $p=0.123$; **Figure 11f**,

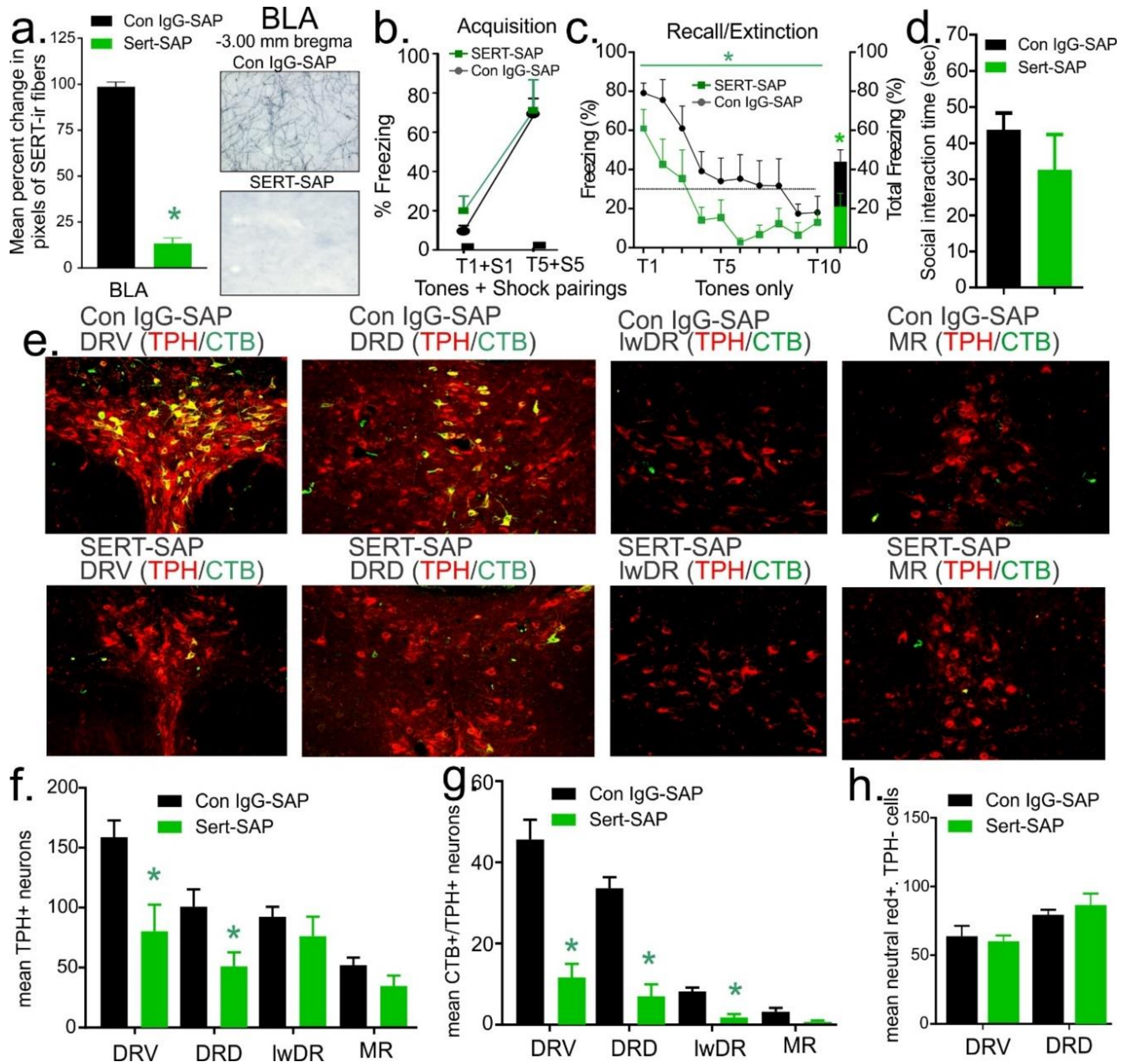


Figure 11 – Effects of bilateral injections of anti-serotonin transporter- (SERT-) or control (IgG-) saporin (SAP) into the basolateral amygdala (BLA) on anxiety-related behavior, fear acquisition, and fear recall/extinction. **(a)** reduced local $SERT^+$ fibers in BLA, * indicates $p < 0.001$ (BLA) in a two-tailed unpaired *t*-test (see representative photos of BLA to the right); **(b)** Intra-BLA SERT-SAP did not alter conditioned fear freezing following 5 tone (T) + shock (S) pairings during fear acquisition session, but **(c)** did reduce freezing response when presented with the tone (T) only during recall/extinction session (overall treatment effect). **(d)** Intra-BLA SERT-SAP had no effect on social interaction time. Injecting a retrograde tracer (Cholera Toxin B subunit: CTB) bilaterally into the BLA at the end of behavioral experiments induced co-localization of CTB (Alexa fluor 488, green) in midbrain serotonergic neurons (TPH, Cy3, red) of control IgG-SAP injected rats in **(e)**, which shows fluorescent photomicrographs from different division of dorsal (DR) and median Raphe (MR) nuclei with top row being from control IgG-SAP injected rats and bottom from SERT-SAP injected rats. **(f)** Reduced total number of tryptophan hydroxylase $(TPH)^+$ neurons in the ventral (DRV) and dorsal (DRD), but not in the lateral wings (lwDR) divisions of the DR, nor the MR; **(g)** and reduced number of CTB^+/TPH^+ neurons in the DRV,

DRD, and lwDR, but not MR. **(h)** In contrast, intra-BLA SERT-SAP did not alter the number of neutral red$^+$ cells that were not colocalized with TPH in neither the DRV ($p = 0.716$) nor DRD ($p = 0.398$). n's per group were 7 IgG-SAP and 5 SERT-SAP for all panels except part **g**, which n=5 each. Bars represent means and standard errors of the means (SEM). Abbreviations: mlf, medial longitudinal fasciculus; Aq., cerebral aqueduct.

n=7 IgG-SAP, 5 SERT-SAP for all). SERT-SAP injections into the BLA did not change the total number of CTB$^+$/TPH$^+$ neurons in MR ($t_{(8)}$=2.2, p=0.0581), but led to mild decreases in the lwDR ($t_{(8)}$=4.6, p<0.005), and greater reductions occurring the DRD ($t_{(8)}$=5.9, p<0.001) and DRV ($t_{(8)}$=5.4, p<0.001; **Figure 11g**, n=5 each, two control IgG-Sap rats lost cap after behavior so no CTB was able to be injected). SERT-SAP did not alter the number of TPH- neutral red-expressing cells neither in the DRD ($t_{(10)}$=3.9, p=0.136) nor in the DRV ($t_{(10)}$=4.6, p=0.716, n=7 IgG-SAP, 5 SERT-SAP, **Figure 11h**).

3.2.3. Experiment 4: Effects of optogenetic excitation of DR-BLA projections on behavior and neural network

Using an intersectional genetics approach, we injected CAV-CMV-Cre bilaterally into the BLA, then either a Cre-dependent control (AAV-EF1a-DIO-eYFP) or the channelrhodopsin-expressing (AAV-EF1a-DIO-ChR2-eYFP) constructs into the midline DR. In animals injected with control construct we observed strong co-localization of eYFP with TPH$^+$ neuron in DRV and some in DRD (Pearson correlation coefficient 0.85) with almost none in lwDR (**Figure 12a**, n=7, photo on the left) or MR (not shown). In the ChR2-injected animals we observed ChR2-eYFP expression within fibers in DRV, DRD (**Figure 12a**, n=8, photo to the right), BLA (**Figure 12b**, photo on the left, n=8) that co-localized with SERT$^+$ fibers (**Figure 12b**, photo on the right, Pearson correlation coefficient 0.95). Wireless optogenetic excitation of the midline DR did not alter fear acquisition [i.e., there

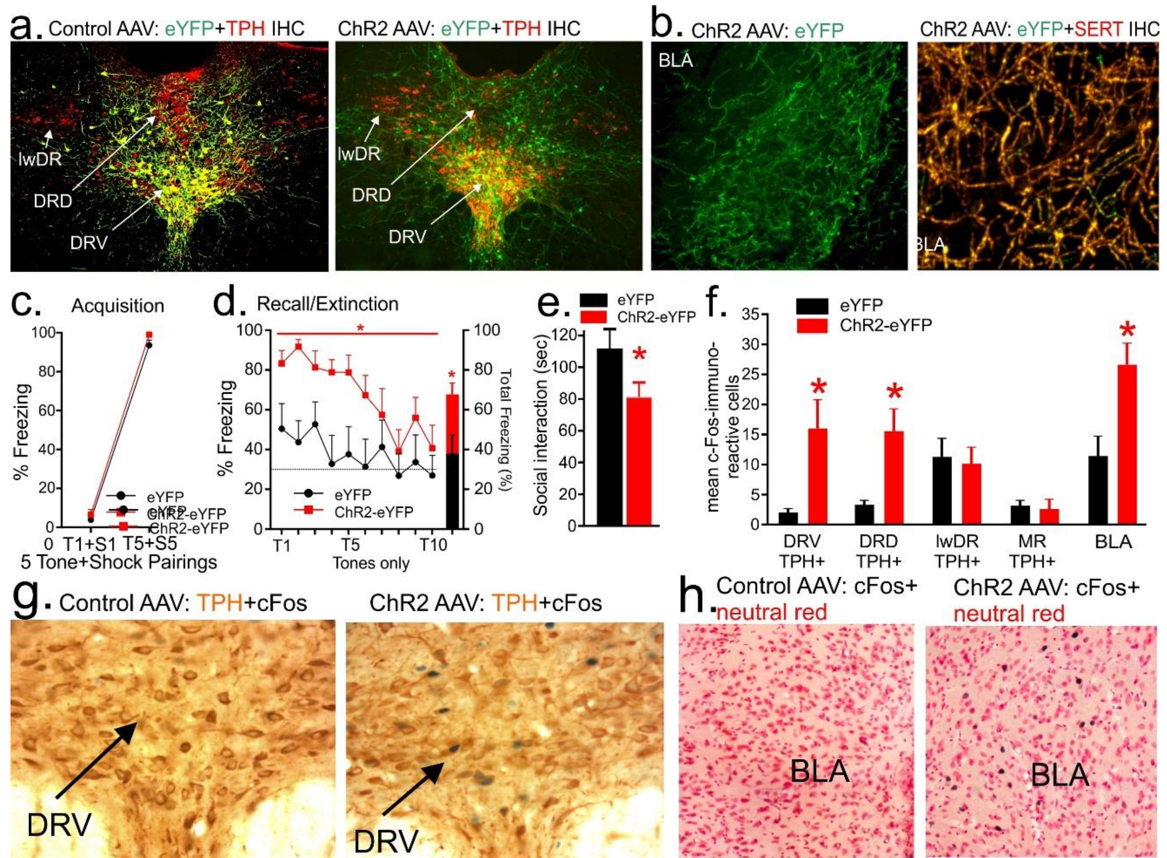


Figure 12 – Bilateral injections of CAV-CMV-Cre into the basolateral amygdala (BLA) and unilateral injections of AAV-EF1a-DiO-ChR2-eYFP into the dorsal/ventral divisions (DRD/DRV) of dorsal Raphe (DR) and effects of optical stimulation of cell bodies in the DR. **(a)** Cre-dependent expression of eYFP (green) within soma of tryptophan hydroxylase $(TPH)^+$ neurons in the DRD and DRV (Cy3, red, photo on the left) and also ChR2 (eYFP, green) on terminals in the DRD/DRV near TPH^+ neurons (Cy3, red, photo on the right). **(b)** Photo represents ChR2-expressing fibers (eYFP, green) in the BLA (photo on the left) that are also co-localized with serotonin transporter (SERT, Cy3, red, photo on the right). Wireless optogenetic excitation of DRD/DRV **(c)** did not alter conditioned fear freezing following 5 tone (T) + shock (S) pairings on fear acquisition session; **(d)** but did increase freezing response when presented with the tone (T) only during recall/extinction session (* indicates $p<0.05$, ChR2 main effect); **(e)** reduced social interaction time; and **(f)** increased cFos within TPH^+ neurons in the DRV and DRD, but not lateral wings (lwDR) or median Raphe (MR), and increased cFos in the BLA (* indicates $p<0.05$). Line and bar graphs represent means and standard errors of the means (SEM). **(g)** Representative photomicrographs of nuclear cFos (blue/black chromogen) and cytoplasmic TPH (orange brown chromogen) immunohistochemistry in the DRV of a control AAV (left) and the ChR2 AAV (right). **(h)** Representative photomicrographs of nuclear cFos (blue/black chromogen) and cytoplasmic neutral red staining in the BLA of a control AAV (left) and the ChR2 AAV (right).

was no treatment x time interaction ($F_{(1,13)}$=0.3, p=0.565), or main effect of treatment ($F_{(1,13)}$=3.5, p=0.084, **Figure 12c**, n=7 eYFP, 8 ChR2), nor consolidation ($F_{(4,52)}$=0.8, p=0.543 and $F_{(1,13)}$=3.8, p=0.072, not shown), but it did enhance overall freezing during fear recall/extinction (ChR2 main effect: line graph, $F_{(1,13)}$=7.8, p=0.015, bar graph $t_{(13)}$=2.8, p=0.023), but there was no treatment x time interaction ($F_{(4,52)}$=0.8, p=0.538, **Figure 12d**, n=7 eYFP, 8 ChR2). Here, blue light stimulation in the DRV decreased SI time ($t_{(12)}$=2.59, p=0.023, **Figure 12e**, n=7 each), and also increased cellular cFos response in TPH^+ neurons in the DRD ($t_{(12)}$=3.2, p=0.0038) and DRV ($t_{(12)}$=2.9, p=0.0066, **Figure 12f-g**), but not in TPH^+ neurons in the lwDR ($t_{(12)}$=0.3, p=0.785) or MR ($t_{(12)}$=0.3, p=0.763, **Figure 12f**, n=7 each, one ChR2 rat was removed due to poor staining). The ChR2-expressing group also had increased cFos in the BLA ($t_{(13)}$=3.1, p=0.0042, **Figure 12f**, **h**, n=7 eYFP, 8 ChR2).

3.3. Discussion

Overall, our major findings support the hypothesis that serotonergic neurons within DRD/DRV that project to the amygdala nuclei may enhance anxiety-related and conditioned fear behaviors.

In our first experiment we confirmed that injections of a retrograde tracer into the BLA resulted in many CTB^+/TPH^+ neurons in the DRD and DRV, but few in lwDR and MR, with approximately 92% of co-localization between CTB^+ and TPH^+. Our data are consistent with Hale and colleagues (Hale et al., 2008) that demonstrated substantial co-localization of DRV and DRD serotoninergic neurons after injecting the retrograde tracer CTB into the BLA, and thus reproducible. Our anterograde tracer into the midline

DRD/DRV in experiment 2 revealed a high density of fibers in the BLA, and this pattern further confirmed by referencing the connectome of the mouse brain in the Allen Brain Atlas (Oh et al., 2014), where injections of a Cre-dependent AAV with a fluorescent reporter into the midline DR in a SERT-Cre mouse (Gong et al., 2007) resulted in dense fibers within the BLA. Even though it was already expected, the similar pattern of anterograde tracing provided us insight on how conserved the DR projections to the BLA are between these two species, an important issue that should be considered when doing preclinical research aiming for translational implications. Collectively, the combination of our anterograde and retrograde tracing studies allowed us to determine not only which site within the BLA to pursue for our following gain and loss of function experiments, but more importantly it showed consistency with the literature, an integral part of research guidelines encouraged by NIH to strengthen published studies.

In our third experiment, we wanted to confirm previous study in which we injected the serotonin neurotoxin 5,7-DHT into the BLA, resulting in reduced local 5-HT concentration and anxiety- and fear-related behavioral responses as assessed by reductions in SI time and in conditioned fear behaviors, respectively (Johnson et al., 2015b). In order to do this, we injected a SERT-SAP into the BLA, which did not alter SI behavior but did attenuate fear-associated freezing during consolidation and extinction compared to control group injected with IgG-SAP. We confirmed that the SERT-SAP group had ~90% reduction in the density of SERT immunostaining in the BLA and ~10% reduction in the density of SERT immunostaining in the perifornical hypothalamus, a structure that receives less innervation arising from midline DR (Muzerelle et al., 2016), thus a control site. Injection of SERT-SAP into the BLA also reduced the total number of TPH$^+$ neurons in the DRV

and DRD, but not in the lwDR or MR. When we assessed CTB^+/TPH^+ neurons we saw a significant reduction in the DRV and DRD, but also in the few CTB^+/TPH^+ neurons in the lwDR, but not the MR. We also noted that only ~8% of CTB-expressing cells in the DR and MR were non-serotonergic. Overall, this loss-of-function approach is consistent with our previous work (Johnson et al., 2015b) and previous 5,7-DHT study in which reductions of serotonin signaling in the amygdala following contextual fear conditioning reduced recall of fear-associated freezing (Izumi et al., 2012). Although we did not see a significant reduction in SI here, we (Johnson et al., 2015b) and others (File et al., 1981) did see significant reductions in SI following 5,7-DHT injections into the amygdala.

In light of our findings that 92% of neurons in the DR that project to the BLA are serotonergic and to further determine this pathway's role in regulating anxiety- and fear-related behaviors, in experiment 4 we utilized intersectional genetics by first injecting into the BLA a retrogradely trafficked CAV that expresses Cre-recombinase (Soudais et al., 2001) which allowed us to selectively introduce a Cre-dependent ChR2 (or its eYFP control) into BLA-projecting DR neurons (DRD/DRV-BLA projections). After immunostaining for TPH in control rats, we determined that all eYFP was co-localized with TPH, with a large expression in the DRV, a few labeled neurons in the DRD, and almost none in the lwDR or the MR. In the animals injected with ChR2 AAV, the eYFP expression was primarily located in fibers in the midline DR and BLA. Altogether, these data further confirmed our anatomical results from our first three experiments, i.e. the tracing studies and the SAP injections. The terminals within the BLA were almost entirely co-localized with SERT, and were, thus, serotonergic. Optogenetic excitation of ChR2-eYFP, but not eYFP control, in the midline DR did not alter fear acquisition nor consolidation, but did increase

freezing during initial tone presentation during recall/extinction session. The stimulation also led to anxiety-associated reduction in SI time. We then verified that optogenetic stimulation increased cellular cFos response within serotonergic neurons in the DRV and DRD and also within the cells in the BLA.

Overall, the aforementioned data support the hypothesis that serotonergic neurons in the DRD/DRV that project to the BLA enhance anxiety-like behavioral and conditioned fear response. It is important to re-emphasize that these experiments lesioned or optogenetically excited 5-HT neurons in the midline, of which many are most likely express glutamate as a co-transmitter (Calizo et al., 2011). Baratta and colleagues injected a Cre-dependent Arch-GFP AAV into the midline DR of SERT-Cre mice and observed that optogenetic inhibition of the DR attenuated only stress-enhanced fear (Baratta et al., 2016). The lack of effect on normal cue-induced fear may have been due to use of a reduced shock intensity or species differences. Future studies are needed to understand how exactly lesioning or exciting DRD/DRV 5-HT projections to the BLA is altering electrophysiological activity of BLA projection neurons that control anxiety- and fear-related behavioral responses. In regards to what is known about 5-HT effects in the BLA there are two factors to consider: 5-HT induces both excitatory and inhibitory actions depending on the receptor subtype and 5-HT receptor subtypes are expressed on both GABAergic interneurons and glutamatergic projection neurons (McDonald and Mascagni, 2007). Glutamatergic principal neurons that enhance anxiety and conditioned fear responses have high expression of excitatory 5-HT_{2C} receptors in the dorsal part of the BLA [i.e., lateral amygdala; (Greenwood et al., 2012)] and 5-HT excites these neurons via the 5-HT_{2C} receptor (Yamamoto et al., 2014). Additionally, acute injections of 5-HT, an SSRI, or a 5-HT_{2C}

receptor agonist into the BLA induce anxiogenic responses (Vicente and Zangrossi, 2012). Yet, application of 5-HT in the BLA region initially produces inhibitory responses by depolarizing GABAergic interneurons (Bocchio et al., 2015; Rainnie, 1999) which have high expression of excitatory 5-HT_{2A} receptors (McDonald and Mascagni, 2007) and play a role in the depolarization (Bocchio et al., 2015). However, there is previously mentioned evidence that stress- and fear-related conditions can produce prolonged release of 5-HT that may lead to loss of local inhibition (Amat et al., 1998; Zanoveli et al., 2009), and there is evidence that this can potentially reduce local GABA inhibition and produce excitation of glutamatergic neurons. For instance, stress exposure can induce downregulation of 5-HT_{2A} receptor expression in the BLA and reduce 5-HT's effects on local GABAergic tone (Jiang et al., 2009). This could lead to excitation of glutamatergic neurons, which play a critical role in enhancing fear conditioning. This hypothesis is supported by previously mentioned studies where initial treatment of rats with SSRIs increases extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001), enhances initial fear-related freezing response in rodents (Ravinder et al., 2013), and enhances conditioned fear in humans (Grillon et al., 2007).

3.4. Conclusions

These data support the role of DR 5-HT projections to the BLA in the modulation of anxiety-like behavior and conditioned fear freezing response. Furthermore, our data using intersectional genetics approach and SERT-SAP are consistent with previous experiments where increasing or depleting 5-HT levels in the BLA region respectively enhances or diminishes conditioned fear behavior and further support the hypothesis that increased

5-HT activity within the amygdala may be an important mechanism in the pathophysiology of severe anxiety disorders, as well as trauma-related disorders, such as PTSD (Wellman et al., 2007; Zanoveli et al., 2009).

Chapter IV: Identification of a Novel Perifornical Hypothalamic Projecting Serotonergic System that Inhibits Innate Panic and Conditioned Fear Responses

4.1. Introduction of Chapter IV

Panic is an innate adaptive response to cope with an imminent threat with an integrated pattern of behaviors (e.g., fighting or fleeing), and also include cardiovascular, respiratory, and endocrine changes. The seminal work of Hess and Brugger (Hess and Brugger, 1943), determined that the perifornical hypothalamic region (PeFR) was a putative panic generating site where electrical stimulation in cats produced panic associated defense reactions (e.g., piloerection, hissing, and arching of back). Subsequent work in rodents determined that pharmacological stimulation or disinhibition of the PeFR increases behavioral (running and escape) components of the "fight-or-flight" response that occur alongside increased physiological [blood pressure (BP), tachycardia, thermal changes, and hyperventilation) (Anderson and DiMicco, 1990; Di Scala et al., 1984; Duan et al., 1996; Markgraf et al., 1991; Olds and Olds, 1962; Shekhar et al., 1990; Shekhar and DiMicco, 1987; Soltis and DiMicco, 1992). In human, stimulating the PeFR produces self-reports that they fear they are dying or having a panic attack (PA) as well as similar physiological responses (i.e., tachycardia, increased BP, thermal sensations, and hyperventilation) (Rasche et al., 2006; Schoenen et al., 2005).

An interesting candidate underlying the panic responses within the PeFR is the orexin (OX) neuropeptide synthesizing neuronal population almost exclusive to this region (Peyron et al., 1998b; Thannickal et al., 2000). These OX producing neurons were found to be critical for the expression of anxiety- and panic-like responses in rats (Johnson et al.,

2010) as panic-associated behaviors and cardiovascular responses elicited by 20% CO_2 inhalation (a suffocation inducing stimuli) were attenuated upon pharmacological inhibition of the OX1, but not OX2 receptors (Johnson et al., 2015a, 2012c). More importantly, OX was found to be elevated in the cerebrospinal fluid of human subjects with panic when compared to controls (Johnson et al., 2010), and in a recent clinical trial, the dual OX receptor antagonist suvorexant was found to improve anxiety symptoms in patients suffering from insomnia (Nakamura and Nagamine, 2017).

Serotonin (5-HT) has long been shown to play a critical role in anxiety and psychological trauma related disorders, such as panic (PD) and post-traumatic stress disorders, respectively [(for review, see (Hale et al., 2012; Hood et al., 2016)]. Within the PeFR, 5-HT appears to inhibit escape behaviors from local excitation or disinhibition and also stress induced endocrine responses via the 5-HT_{1A} receptor. This is suggestive that 5-HT inhibits escape behaviors associated with panic but did not assess other aspects of an integrative panic response such as cardioexcitation, thermal changes and only assessed 5-HT's role via the 5-HT_{1A} and 5-HT_{2A} receptors (da Silva et al., 2011; Nascimento et al., 2014). In support of a panicolytic role for the 5-HT_{1A} receptor, OX neurons express 5-HT_{1A} receptor (Collin et al., 2002; Muraki et al., 2004) and receive dense projections from the serotonergic system (Ljubic-Thibal et al., 1999; Muzerelle et al., 2016). Additionally, electrophysiological studies showed that 5-HT directly inhibits OX neurons, an effect that is blocked with a 5-HT_{1A} receptor antagonist (Muraki et al., 2004).

However, there is a gap in knowledge in serotonergic regulation of the PeFR in the context of panic-associated behaviors and physiological responses since site specific injections of drugs ignore circuit-specific neurotransmission and its physiological properties

that occur *in vivo*. Therefore, we hypothesize that optogenetic excitation of serotonergic fibers at the PeFR originating from the DR/MR will attenuate the physiological and behavioral components of panic-like responses induced by a panicogenic/suffocation related stimuli (7.5% and 20% CO_2 exposure). We also hypothesized that selective lesioning of 5-HT fibers in the PeFR would lead to exacerbated physiological and behavioral components of panic-like responses induced by a panicogenic/suffocation related stimuli (7.5% and 20% CO2 exposure). Finally, since the OX PeFR system projects to the amygdala (Peyron et al., 1998b) where it enhances fear conditioned behaviors (Dustrude et al., 2018; Flores et al., 2014), we also hypothesized that PeFR-projecting serotonergic system would play an inhibitory role on conditioned fear behaviors.

In order to test these hypotheses, we used circuit-based loss- and gain-of-function studies to investigate the role of 5-HT originating from the DR/MR in the context of innate panic-associated behaviors and physiology and also in conditioned fear behaviors. To further confirm that activating the PeFR not only induced panic-associated behaviors and physiology, but also conditioned avoidance, we utilized wireless optogenetics to excite glutamatergic neurons in the PeFR. We then utilized a gain-of-function approach with wireless optogenetic stimulation to selectively activate the PeFR-projecting serotonergic system while assessing innate anxiety- and panic-associated physiology and behaviors. Finally, in our loss-of-function study we first injected a selective anti-5-HT transporter-saporin toxin (SERT-SAP) into the PeFR and assessed anxiety-like and conditioned fear responses, then injected a retrograde tracer into the same site to confirm lesioning of the projecting Raphe nuclei.

4.2. Results

4.2.1. ***Experiment 1:*** *Effects of Optogenetic Stimulation of PeF Glutamatergic Neurons*

Our immunohistochemistry showed bright green fluorophore colocalized with OX^+ neurons within the PeFR (Pearson correlation coefficient 0.63, **Figure 13b**). Optogenetic activation of glutamatergic cell bodies of ChR2-injected rats in the PeF led to robust increases BP (virus x time interaction $F_{(30,240)}=14.26$, $p<0.0001$), heart rate (HR, $F_{(30,240)}=6.47$, $p<0.0001$, **Figure 13c**), and general motor activity (virus x time interaction $F_{(30,240)}=3.37$, $p<0.0001$, **Figure 13d**) qualitatively analyzed as aversive behaviors [i.e. long periods of exophthalmos ($p<0.0001$) and immobility ($p<0.0001$) followed by running ($p=0.007$), **Figure 13d**, inset]. Optogenetic stimulation also virtually abolished SI time (two-tailed, unpaired Student's t test, $p<0.0001$, **Figure 13e**) without promoting aggressive behavior. To further test whether ChR2 stimulation could induce conditioned aversive memory, we paired optical stimulation to one side of a two-chamber apparatus and observed avoidance from the stimulation chamber (virus effect $F_{(1,8)}=22.91$, $p=0.001$) during optical stimulation ($p=0.001$), short- ($p=0.01$), and long-term ($p=0.01$, n=5 each, **Figure 13f**) sessions. Lastly, optical stimulation 90 min prior to euthanasia induced a marked increase in cFos in OX^+ neurons (Mann-Whitney test, $p=0.007$) (**Figure 13g**).

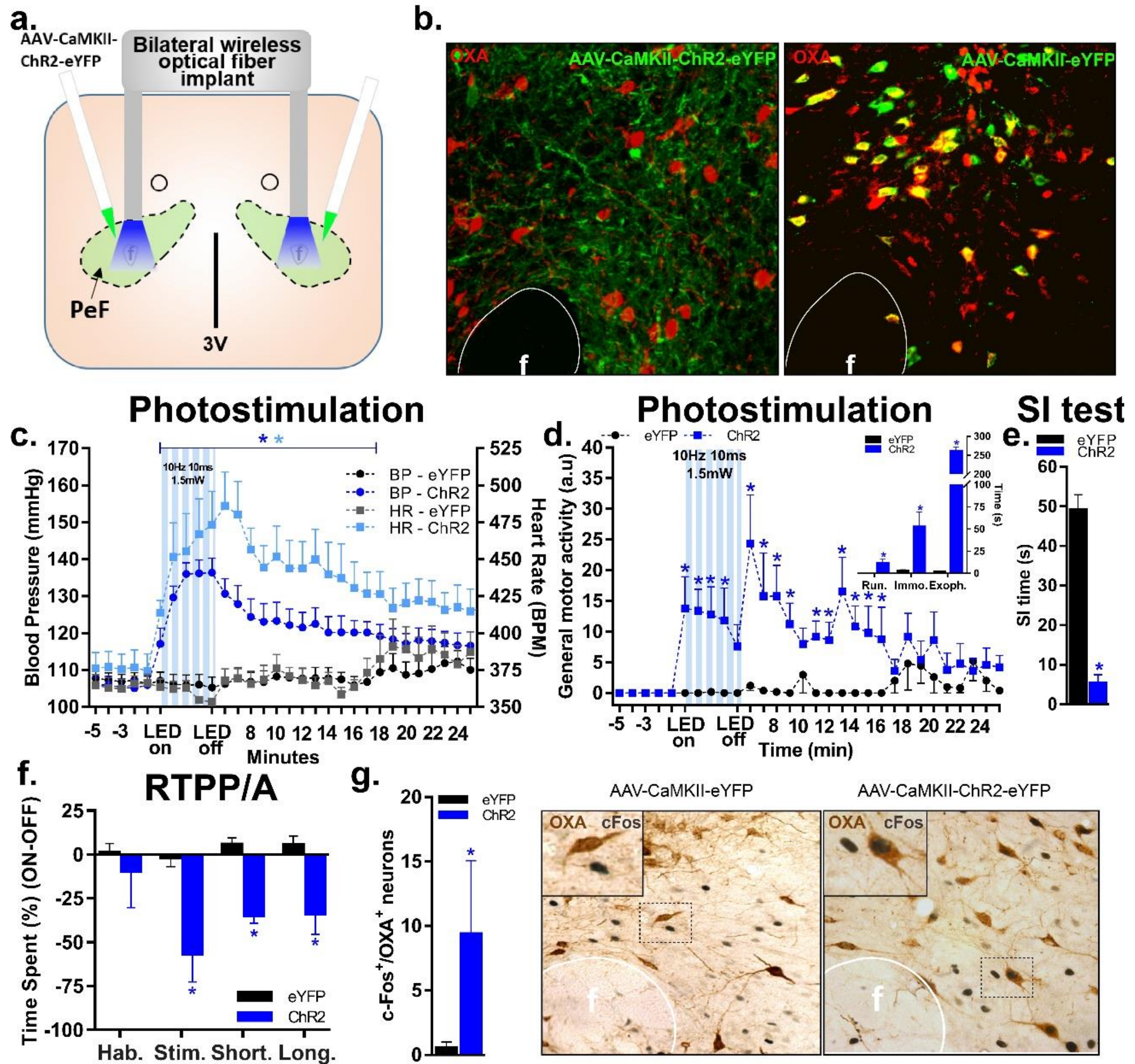


Figure 13 – Optogenetic stimulation of glutamatergic cell bodies in the perifornical hypothalamus (PeF) elicits escape and cardiovascular excitation that resembles panic. **(a)** Schematic illustration of rats bilaterally injected with AAV5-CaMKIIa-ChR2-eYFP or its control AAV-CaMKIIa-eYFP (not illustrated) into the PeF, and photostimulation with bilateral wireless optical fiber implants over the PeF. **(b)** Representation of confocal images from rats expressing eYFP fibers (ChR2, green, left panel) and soma (control, green, right panel) and orexin A peptide (OXA)-expressing neurons (red, Alexa 640). Note co-localization between OXA^+ and $eYFP^+$ soma in control group (Pearson correlation coefficient 0.63). **(c)** ChR2 rats had a significant increase in blood pressure (BP, circle signs, left y axis), heart rate (HR, square signs, right y axis), and **(d)** general motor activity (circle and square signs, *, $p<0.01$, between subjects differences using a Fisher's LSD post hoc test) and its qualitative behavioral responses (inset two-tailed, unpaired Student's t test, *, $p<0.01$), i.e. running (Run.), immobility (Immo.) and exophthalmos (Exoph.), during optogenetic stimulation (10 Hz, 10 ms, 1.5 mW, LED on-LED off, 5 min). Data obtained from freely moving rats surgically implanted with radiotelemetry probes. Note stable baselines for both BP, HR, and general motor activity (t-5 to t-1). **(e)** ChR2 rats had significant less

social interaction (SI) time test during photostimulation of the PeF in the SI test (two-tailed, unpaired Student's t test, *, $p<0.0001$). **(f)** ChR2 rats had a significant lower difference score (percentage time spent in stimulation side minus percentage time spent in non-stimulation side, *, $p<0.05$, between subjects differences using a Fisher's LSD post hoc test) than the control group during optical stimulation (Stim.), short- (Short.), and long-term (Long.) sessions, but not during habituation (Hab.) in the real-time place preference/avoidance (RTPP/A) test. **(g)** ChR2 rats (right panel) had significant more activation of the immediate early gene cFos colocalized with OXA^+ neurons (Mann-Whitney test, *, $p=0.007$) than control group (left panel). Abbreviations: 3V, third ventricle; f, fornix;

4.2.2. ***Experiment 2:*** *Anatomical Confirmation of PeF-projecting Serotonergic Systems Utilizing CTB.*

Our experiment 2 showed that injections of the retrograde tracer CTB into the PeF (**a**) led to CTB expression in TPH^+ neurons in the lwDR and MR (**Figure 14e-f**), but few in the DRD or DRV (**Figure 14c-d**, see topography of midbrain TPH^+ neurons in **Figure 14b**, n=4) with only 5-8% of CTB being expressed in TPH- neurons. This study revealed that 92-95% of PeF-projecting neurons within lwDR/MR express TPH, thus are serotonergic.

4.2.3. ***Experiment 3:*** *Effects of Optogenetic Stimulation of PeF-Projecting lwDR/MR Fibers on Physiology and Behaviors of Anxiety- and Panic-Associated Challenges*

Using the combination of a two-virus approach, we took advantage of the highly selective serotonergic projections from the lwDR/MR to the PeF (lwDR/MR→PeF) and injected the retrogradely trafficked CAV-CMV-Cre bilaterally into the PeF and either a Cre-dependent eYFP control (AAV-EF1a-DIO-eYFP) or active (Cre-dependent) ChR2 (AAV-EF1a-DIO-ChR2-eYFP) into the lwDR/MR. We used this approach to activate the lwDR/MR→PeF ChR2-expressing fibers (lwDR/MR→PeF:ChR2 or its control :eYFP

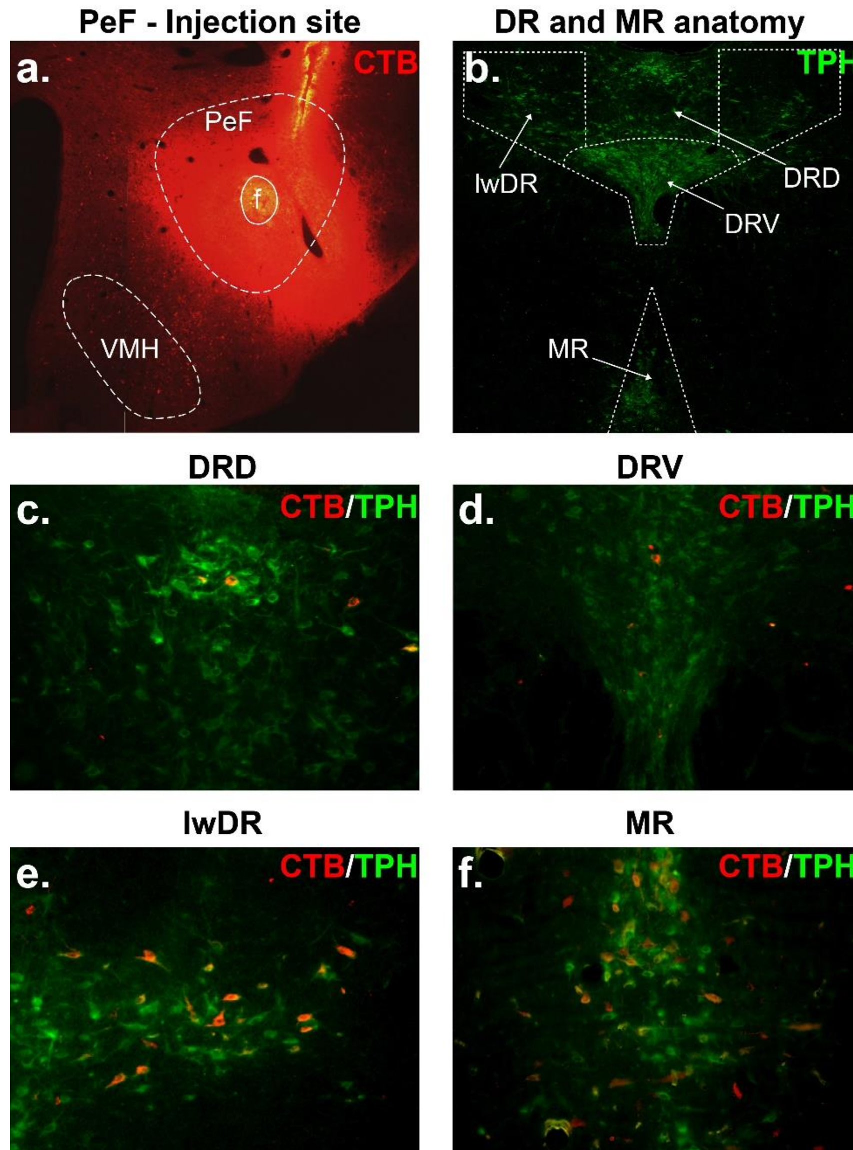
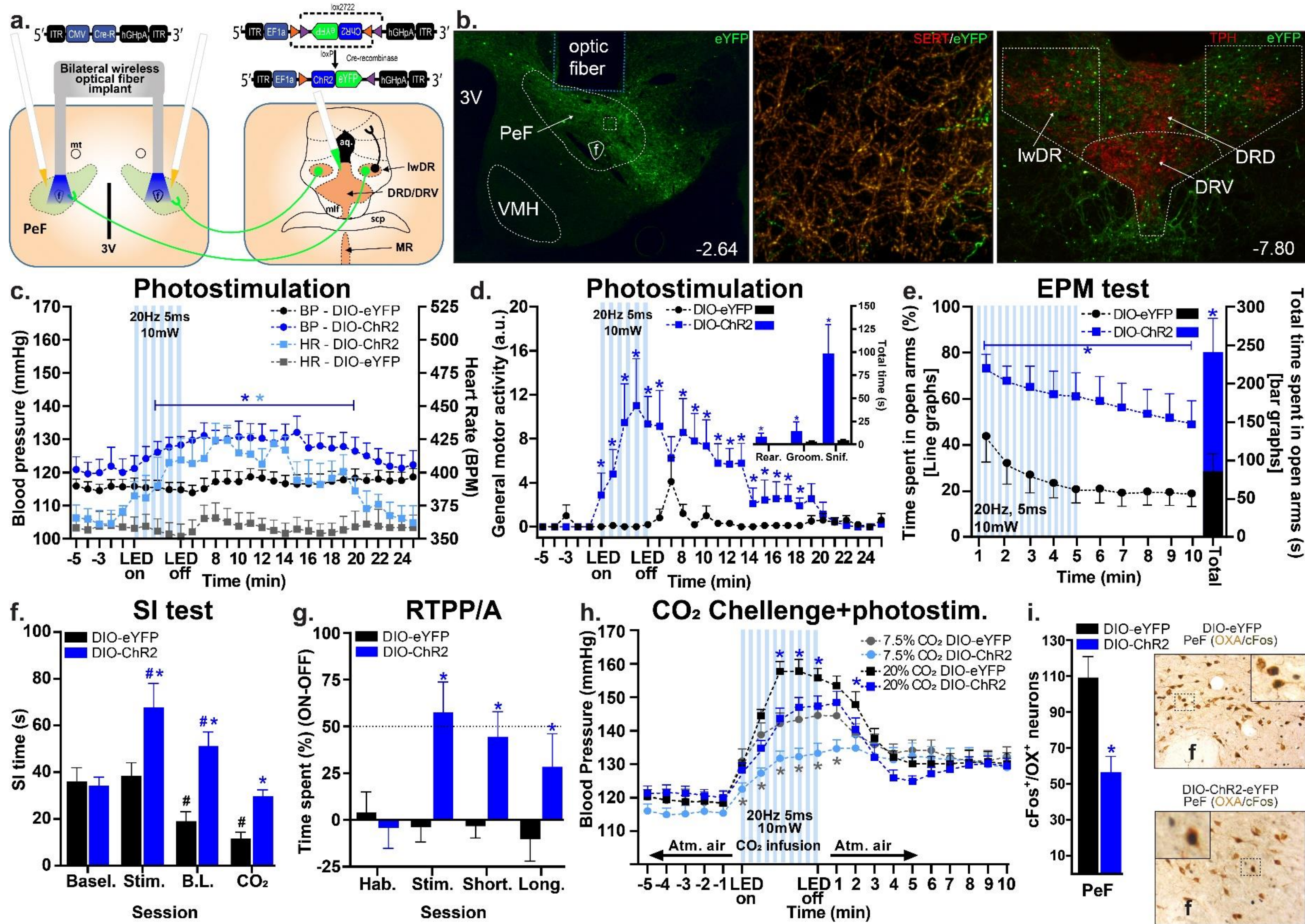


Figure 14 – Retrograde tracing from the perifornical hypothalamus (PeF) using Cholera toxin B subunit (CTB). **(a)** Injection site illustrating CTB injection (Alexa 567, red) injection into the PeF. **(b)** Topography of tryptophan hydroxylase (TPH, Alexa 488, green) immunoreactive serotonergic neurons in the midbrain Raphe nuclei [i.e., median Raphe (MR) nucleus and dorsal (DRD), ventral (DRV) and lateral wing (lwDR) divisions of the dorsal Raphe (DR) nucleus] In panels **(c)** through **(e)** are immunohistochemistry reactions against TPH and CTB highlighting mostly PeF-projecting serotonergic neurons originating from **(e)** lwDR and **(f)** MR, but only a few from **(c)** DRD and **(d)** DRV. Abbreviations: f, fornix; VMH, ventromedial hypothalamus.

from this point forward, **Figure 15a**). In animals injected with active ChR2, we observed eYFP$^+$ fibers in the PeF (**Figure 15b**, photo on the left) that co-localized with SERT signal (**Figure 15b**, photo in the middle). Expression of eYFP$^+$ neurons were mostly localized in

the lwDR (**Figure 15b**, photo on the right) and MR (not shown), with almost none in DRD/DRV. Surprisingly, optogenetic stimulation of lwDR/MR→PeF:ChR2 animals led to a mild increase in BP (virus effect, $F_{(1,17)}=4.2$, $p=0.05$), HR ($F_{(1,17)}=12.83$, $p=0.002$, **Figure 15c**), and locomotion [i.e. general exploratory/self-directed behaviors: sniffing ($p=0.008$), rearing ($p=0.02$), and grooming ($p=0.02$), n=10 DIO-eYFP, 9 DIO-ChR2, **Figure 15d**]. Next, optical stimulation of lwDR/MR→PeF:ChR2 paired to open arm exploration in the EPM greatly increased the total time spent in this region of the apparatus not only during, but also after paired optogenetic stimulation (virus effect $F_{(1,14)}=12.34$, $p=0.003$, **Figure 15e**). The SI testing was carried out in four different sessions tested throughout four consecutive days: baseline (n=9 eYFP, 10 ChR2), optical stimulation (n=9 each), bright light (n=9 each), and 20% CO_2 (n=7 each) challenges (n's differ between sessions due repeated disconnections of optogenetic stimulator during SI testing). This procedure resulted in a significant virus ($F_{(1,61)}=23.8$, $p<0.0001$), session ($F_{(3,61)}=10.42$, $p<0.0001$), and interaction ($F_{(3,61)}=4.08$, $p=0.01$) effects, with optical stimulation of ChR2 increasing SI time for all sessions except baseline (**Figure 15f**). Exposure to RTPP/A led to a strong place preference behavior of ChR2 group [virus x session ($F_{(3,39)}=3.75$, $p=0.01$)] during optical stimulation ($p=0.001$), 45 min ($p=0.01$), and 24 hour ($p=0.03$) sessions (n=7 DIO-eYFP, 8 DIO-ChR2; **Figure 15g**). Lastly, optical stimulation of lwDR/MR→PeF:ChR2 attenuated CO_2-induced (7.5 and 20%) increases in BP compared to eYFP (virus x time interaction $F_{(19,285)}=2.61$, $p=0.0003$ and $F_{(19,304)}=2.61$, $p=0.0003$, respectively, **Figure 15h**). After activation of lwDR/MR→PeF fibers, there was decreased cFos responses in in OX^+ neurons in the PeF ($t_{(8)}=3.6$, $p=0.006$, n=5 each, **Figure 15i**)



a.
Bilateral wireless optical fiber implant
PeF
3V
mt
aq.
lwDR
DRD/DRV
mlf
scp
MR
Cre-recombinase
b.
eYFP
optic fiber
3V
PeF
VMH
-2.64
SERT/eYFP
TPH eYFP
lwDR
DRD
DRV
-7.80
c.
Photostimulation
20Hz 5ms 10mW
BP - DIO-eYFP
BP - DIO-ChR2
HR - DIO-ChR2
HR - DIO-eYFP
Blood pressure (mmHg)
Heart Rate (BPM)
LED on
LED off
Time (min)
d.
Photostimulation
20Hz 5ms 10mW
DIO-eYFP
DIO-ChR2
General motor activity (a.u.)
Total time (s)
Rear.
Groom.
Snif.
LED on
LED off
Time (min)
e.
EPM test
DIO-eYFP
DIO-ChR2
Time spent in open arms (%) [Line graphs]
Total time spent in open arms (s) [bar graphs]
20Hz, 5ms 10mW
Time (min)
Total
f.
SI test
DIO-eYFP
DIO-ChR2
SI time (s)
Basel.
Stim.
B.L.
CO₂
Session
g.
RTPP/A
DIO-eYFP
DIO-ChR2
Time spent (%) (ON-OFF)
Hab.
Stim.
Short.
Long.
Session
h.
CO₂ Chellenge+photostim.
7.5% CO₂ DIO-eYFP
7.5% CO₂ DIO-ChR2
20% CO₂ DIO-eYFP
20% CO₂ DIO-ChR2
Blood Pressure (mmHg)
20Hz 5ms 10mW
Atm. air
CO₂ infusion
Atm. air
LED on
LED off
Time (min)
i.
DIO-eYFP
DIO-ChR2
cFos⁺/OX⁺ neurons
PeF
DIO-eYFP PeF (OXA/cFos)
DIO-ChR2-eYFP PeF (OXA/cFos)
f

Figure 15 – In the gain-of-function study, optogenetic stimulation of perifornical hypothalamus (PeF)-projecting serotonergic terminals reduced anxiety- and panic-associated responses and promoted increased exploration. **(a)** Schematic illustration of rats bilaterally injected with AAV5-DIO-ChR2-eYFP or AAV5-DIO-eYFP (not illustrated) into the dorsal Raphe nucleus (DR) and CAV-CMV-Cre into the PeF, and photostimulation with bilateral wireless optical fiber implants over the PeF. **(b)** Representation of confocal images from rats expressing ChR2-eYFP fibers (green, photo on the left) that colocalized with serotonin transporter (SERT, red, Alexa 640, photo in the middle) in the PeF and ChR2-eYFP fibers (green, photo on the right) in the lateral wings (lwDR), and less fibers in the dorsal (DRD) and ventral (DRV) DR with tryptophan hydroxylase $(TPH)^{+}$ neurons (red, Alexa 640). Median Raphe nucleus (MR) is not shown. Numbers indicate distance, in mm, from bregma according to (Paxinos and Watson, 1998). Optogenetic stimulation (20 Hz, 5 ms, 10 mW, LED on-LED off, 5 min) of lwDR/MR→PeF:ChR2 rats induced a significant **(c)** increase in blood pressure (BP, circle signs, left y axis), heart rate (HR, square signs, right y axis) and **(d)** general motor activity (circle and square signs). **(e)** lwDR/MR→PeF:ChR2 rats had increased open arm exploration during (1-5 min) and post (6-10 min, line graphs, left y axis) optogenetic stimulation in the elevate plus-maze (EPM) test (see total time, in s, of open arm exploration in bar graphs and right y axis). Compared to controls, photostimulation of lwDR/MR→PeF:ChR2 rats **(f)** increased social interaction (SI) time during stimulation (Stim.), bright-light (B.L.), and CO_2 sessions [symbols are: *comparison between groups; #within groups compared to baseline (Basel.)]; **(g)** increased the difference score (percentage time spent in stimulation side minus percentage time spent in non-stimulation side) real-time place preference/avoidance (RTPP/A) test; **(h)** attenuated CO_2-induced increases in BP in the CO_2 challenge + photostimulation (photostim.) test; and **(i)** attenuated cFos responses in in orexin $(OX)^{+}$ neurons in the PeF. Graphs in **(c-h)** are two-way ANOVA followed by Fisher's LSD post hoc test, whereas bar graphs in **(c, e,** and **i)** are unpaired Student's t test. * and # are $p<0.05$. Abbreviations: 3V, third ventricle; f, fornix; mt, mammillothalamic tract; OXA, orexin A peptide; VMH, ventromedial hypothalamus.

4.2.4. ***Experiment 4:*** *SERT-SAP Injection into the PeF Selectively Lesions Local SERT Fibers and Their Associated Cell Bodies in the lwDR and MR.*

SERT-SAP injection in the PeF significantly reduced the density of local SERT-positive fibers in the PeF by approximately 42% (two-tailed, unpaired Student's t test, p=0.001), but did not change the density of local amygdala fibers (control site, n=7 each, **Figure 16b**), which receives sparse fibers from PeF-projecting 5-HT neurons (Muzerelle et al., 2016), if any. In control IgG-SAP rats, bilateral injections of CTB into the PeF led

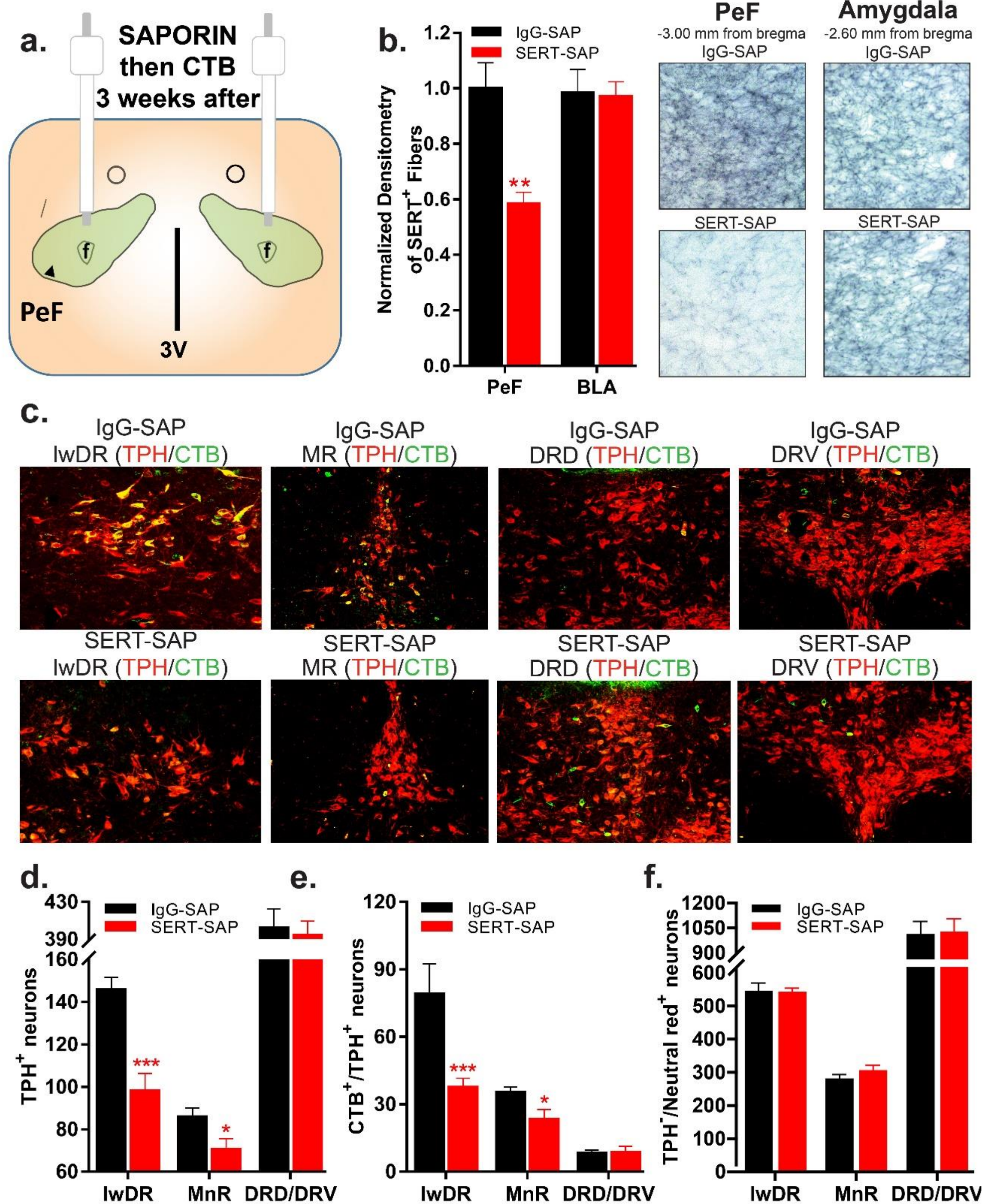


Figure 16 – Injection of saporin (SAP) toxin conjugated to the serotonin transporter antibody (SERT-SAP) lesions local perifornical hypothalamus (PeF) SERT$^+$ fibers and their associated tryptophan hydroxylase (TPH)$^+$ cell bodies the median (MR) and lateral wings (lwDR) division of the dorsal Raphe (DR). **(a)** Schematic illustration of rats bilaterally injected with SERT-SAP or its control IgG-SAP (not illustrated) and the retrograde tracer Cholera toxin b subunit (CTB) into the PeF. **(b)** SERT-SAP injected rats had significant reduced (two-tailed, unpaired Student's t test, *, p=0.001) expression of SERT$^+$ fibers in

the PeF (left lower panel photograph) than IgG-SAP rats (left upper panel photograph), but not in the basolateral amygdala (BLA, right panel photographs). **(c)** SERT-SAP injected rats (bottom row) had decreased total number of TPH^+ [red, Alexa 640, bar graphs in **(d)**] and CTB^+/TPH^+ [CTB green, Alexa 488, bar graphs in **(e)**] neurons in the lateral wings (lwDR) DR and MR, but not in the dorsal (DRD)/ventral (DRV) DR when compared to IgG-SAP group (top row). See topography of MR and divisions of DR in **Figure 14b**. **(f)** SERT-SAP injections did not reduce total number of non-TPH neurons in none of the DR and MR regions with neutral red counter staining [two-tailed, unpaired Student's t test, *, $p<0.05$, and ***, $p<0.0005$ for **(d-f)**]. Abbreviations: 3V, third ventricle; Aq., aqueduct; f, fornix.

to CTB expression in many TPH^+ neurons in the MR and lwDR, but few in DRD and DRV (see topography of midbrain TPH^+ neurons in **Figure 16c** and co-localizations in **Figure 16d**). SERT-SAP injections into the PeF also respectively reduced the total number of single-TPH^+ (**Figure 16e**) and double-CTB^+/TPH^+ (**Figure 16f**) neurons in the MR (p=0.03; p=0.02) and lwDR (p=0.0004; p=0.01), but not in the DRD or DRV (n=7 IgG-SAP, 6 SERT-SAP; one animal excluded due poor TPH staining). No SERT-SAP lesioning effects were observed in non-TPH neurons (neutral red^+ cells, **Figure 16g**).

4.2.5. ***Experiment 5:*** *Effects of SERT-SAP Injections into the PeF on Physiology and Behaviors*

In SERT-SAP lesioned rats, while low CO_2 exposure elicited moderate increases only in BP (treatment effect $F_{(1,12)}=11.01$, p=0.006), higher CO_2 concentration induced robust elevation in both BP (treatment effect $F_{(1,12)}=7.62$, p=0.017 and treatment x time interaction $F_{(19,228)}=3.65$, $p<0.0001$, **Figure 17a**) and locomotor activity (treatment effect $F_{(1,12)}=8.54$, p=0.01, **Figure 17b**). Lesioned rats displayed reduced total SI time (p=0.02, n=7 each, **Figure 17c**), decreased the latency to escape the open arm [i.e. treatment effect ($F_{(1.11)}=7.16$, p=0.021, n=7 IgG-SAP, 6 SERT-SAP; one animal excluded due repeated falls from ETM; **Figure 17d**)], but did not have differences on avoidance in the ETM and OF

behaviors (not shown). Lastly, intra-PeF injections of SERT-SAP did not affect fear acquisition (**Figure 17e**), but did enhance overall freezing responses during fear extinction [i.e., main effect of treatment ($F_{(1,12)}$=23.72, p=0.0004) and treatment x time interaction ($F_{(19,228)}$=2.69, p=0.0003, **Figure 17f**)].

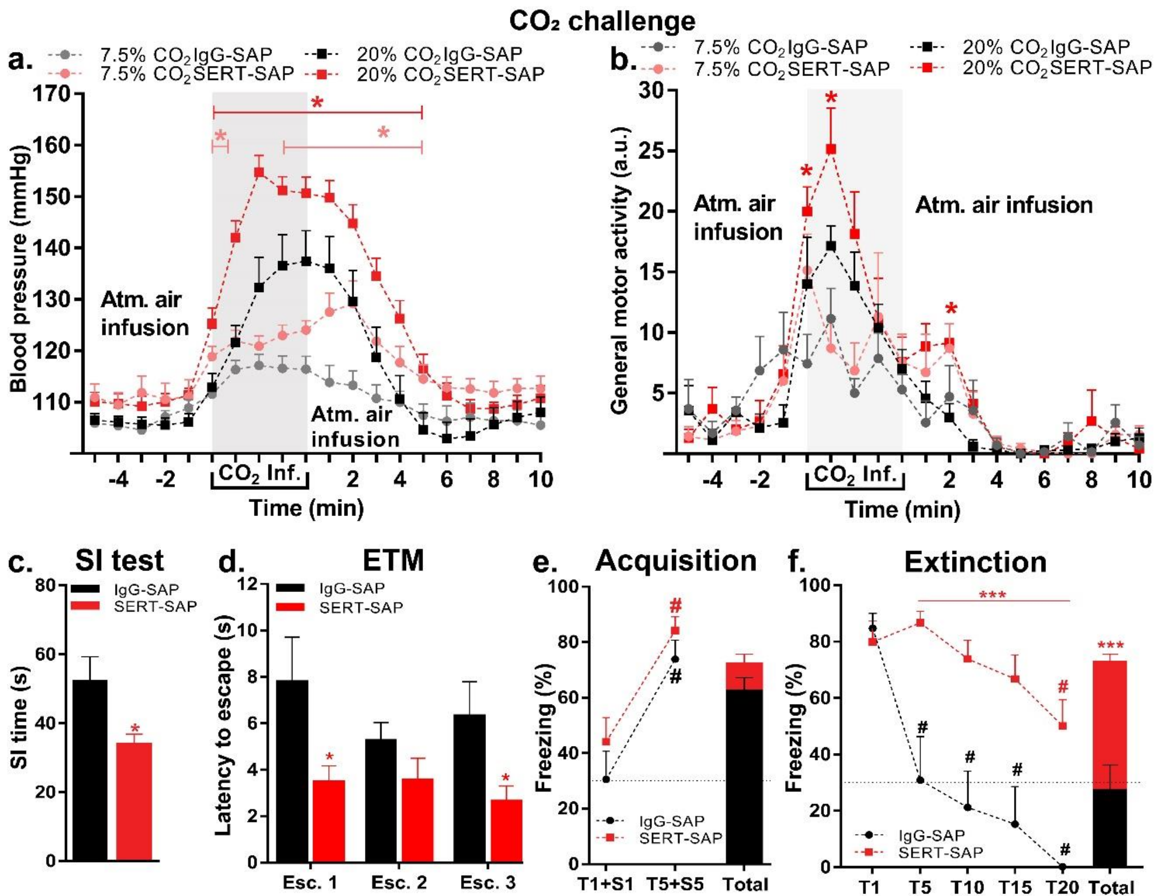


Figure 17 – In the loss-of-function study, injection of saporin (SAP) toxin conjugated to the serotonin transporter antibody (SERT-SAP) into the perifornical hypothalamus (PeF) promotes anxiety-, fear-, and panic-associated responses. SERT-SAP injected rats had: **(a)** increase in blood pressure and **(b)** general motor activity in the CO_2 challenge; **(c)** decrease in social interaction (SI) time in the SI test; **(d)** decrease latency to escape the open arm in the elevated T-maze (ETM); **(e)** normal acquisition of freezing response to tone (T) + shock (S) pairings; and **(f)** increase freezing response to cue-induced conditioned fear and also delayed extinction (*, between groups; #, compared to T1 within groups). Part **(c)** and bar graphs in **(e)** and **(f)** are two-tailed, unpaired Student's t test; bar graph in **(d)** and line graphs **(a-b)** and **(e-f)** are two-way ANOVA followed by Fisher's LSD post hoc test. *, p<0.05; ***, p<0.0005; #, p<0.05. Abbreviations: Atm, atmospheric; Esc., escape response; Inf., CO_2 infusion.

4.3. Discussion

In our first experiment we confirmed that injections of CaMKIIa ChR2 virus into the PeFR led to many $eYFP^+$ fibers in the PeFR region, and high co-localization between OXA and $eYFP^+$ soma with CaMKIIa control virus. Optogenetic stimulation of ChR2 group, but not control, led to robust increases in exophthalmos; escape associated running; decreased social interaction times; as well as cardioexcitation; and increases in core body temperature (data not shown); and in cellular cFos responses in OXA^+ neurons. These responses are similar to those described in the literature utilizing both pharmacological (Freitas et al., 2009; Shekhar and DiMicco, 1987; Ullah et al., 2017, 2015) and electrical stimulation (de Bortoli et al., 2013; Lammers et al., 1988) of the PeFR of rats. The optogenetic excitation of glutamatergic neurons in PeFR also induced an unconditioned and conditioned place aversion, which is consistent with the OX/glutamate PeFR system projecting to the amygdala (Dustrude et al., 2018; Peyron et al., 1998b) where it enhances conditioned fear behaviors (Dustrude et al., 2018; Flores et al., 2014). These effects are not only a direct indication of the averseness of glutamatergic stimulation in the PeF, but also indicate the capacity of this region to organize some forms of emotional learning canonically seen in fear conditioning experiments (LeDoux, 2000; Phelps and LeDoux, 2005). Collectively, optogenetic stimulation of the PeF promotes various orchestrated behavioral and physiological responses that are components of the “fight-or-flight” repertoire, thus reinforcing its role as one of the putative brain regions underlying panic behavior.

As stated in the introduction, there is evidence that the 5-HT_{1A} receptor in the PeFR inhibits escape behaviors from local excitation or disinhibition, but little is known about the role of serotonergic neurotransmission in physiological and behavioral panic responses

or conditioned fear responses in this region. In order to assess the role of serotonergic neurotransmission in these responses in the PeFR we first injected a retrograde tracer CTB into the PeFR and determined that the majority of PeFR-projecting serotonergic neurons originated in the lwDR and MR, with the majority of CTB^+ neurons being serotonergic (92-95%). This is consistent with previous tracing studies revealing that most of the serotonergic neurons innervating the PeFR originate from the lwDR and MR (Ljubic-Thibal et al., 1999; Muzerelle et al., 2016). We then used an intersectional genetics approach where we injected a CAV-CMV-Cre virus into the PeFR and a Cre-dependent AAV-EF1a-DIO-Chr2-eYFP into the lwDR MR. The combination of these two viruses induced expression of ChR2 selectively onto lwDR and MR neurons projecting to PeFR, which colocalized with local serotonergic terminals (i.e., SERT expression). Here, wireless optogenetic excitation of ChR2 fibers in the PeFR that originated from the lwDR and MR resulted in a mild increase in the cardiovascular parameters during optogenetic stimulation. It is noteworthy that the behavior repertoire was qualitatively different from those observed in our first experiment with optogenetic excitation of glutamatergic cell bodies in the PeF. Here, rats displayed investigation-driven behaviors, such as increased exploration, accompanied by sniffing and occasional rearing, and we cannot discard the possibility that these factors may have contributed to the cardioexcitation. In agreement with these results, 5-HT release in the hypothalamus has been linked to the control of wakefulness responses in different stances (Houdouin et al., 1991; Imeri et al., 1994; Yuki C. Saito et al., 2018). Moreover, wireless optogenetic excitation of ChR2 fibers in the PeFR that originated from the lwDR and MR also increased time spent on the open arm; increased social interaction time; and

induced conditioned place preference paired with photostimulation, effect observed 24 hours post-conditioning. These are all consistent with an anxiolytic effect.

We then exposed rats to a suffocation-associated stressor (i.e., 5 min infusion of normoxic 20% CO_2) that elicits core symptoms of a panic attack in humans (e.g., catastrophic fear accompanied by cardiovascular and thermoregulatory responses) (Forsyth et al., 2000). This CO_2 challenge also has predictive validity since fluoxetine and alprazolam, two clinically relevant panicolytic drugs utilized to treat patients with recurrent panic attacks, attenuate escape responses (Spiacci et al., 2018) and panic-associated behaviors and physiological responses (Johnson et al., 2015a, 2012b) elicited by 20% CO_2 inhalation in rodents. Optogenetic excitation of the lwDR/MR fibers that project to the PeFR attenuated CO_2-induced increases in pressor responses and reductions in social interaction time, as well as decreased the number of cFos responses in OXA-producing neurons within the PeFR. Collectively these data support the hypothesis that serotonergic neurotransmission in the PeFR inhibits anxiety avoidance and innate panic behaviors. This hypothesis is further supported in that OX/glutamatergic neurons express the 5-HT_{1A} receptor (Collin et al., 2002; Muraki et al., 2004) and electrophysiological studies show that a 5-HT_{1A} receptor antagonist blocks 5-HT direct inhibition of OX neurons (Muraki et al., 2004) and selective OX1 receptor antagonists attenuate panic associated behavioral, cardiovascular, and thermal responses to 20% CO_2 challenges (Johnson et al., 2015a, 2012b).

In our final experiment we tested the hypothesis that selective lesioning of 5-HT projections to the PeFR would induce a panic and phobia vulnerable state. This hypothesis is based on results from previous experiments, but also that finding that OX neurons send selective projections to the lwDR and MR (Peyron et al., 1998b) where they excite local 5-

HT neurons via the OX1 receptor (Brown et al., 2002). In order to do this we injected SERT-SAP into the PeFR where we observed a 42% reduction in the density of local SERT$^+$ fibers that coincided with selective decrease of the total number of TPH$^+$ neurons in the lwDR and MR, but not in the DRD/DRV (no off-target effects on local non-TPH neutral red cells were observed). This same pattern was confirmed by the highly co-localized CTB$^+$/TPH$^+$ neurons (92-95%), which showed similar amount of reduction in the same Raphe regions. It is noteworthy that no alterations were observed in the SERT$^+$ fibers in the BLA, a structure that receives less innervation arising from lwDR/MR (Muzerelle et al., 2016), hence a control site. Using the same lesioning approach in a different circuit, we were able to induce less extensive reductions of local PeFR 5-HT fibers here when compared to our previous study in the BLA injections of SERT-SAP (~85%) (Johnson et al., 2019), but higher than those reductions in the medulla observed in a previous study (28%) (Nattie et al., 2004). Lesioning of the lwDR/MR→PeFR serotonergic circuit increased innate anxiety- and panic-associated responses measured by reduced SI time, decreased escape latency in the ETM and enhanced general locomotor activity and increased pressor response to CO_2 challenges.

Similar to lesioning 5-HT terminals in the PeFR, chronic disinhibition of GABA synthesis in the PeFR also induces a panic vulnerable state (Johnson et al., 2008; Johnson and Shekhar, 2006; Molosh et al., 2010). OX neurons also express $GABA_A$ receptor subunits and are disinhibited by bicuculline methiodide or inhibited by muscimol, antagonist and agonist of $GABA_A$ receptor, respectively (Eggermann et al., 2003). Along those lines, *in vitro* bath application of 5-HT or optogenetic stimulation of local 5-HT terminals in brain slices produce $GABA_A$-mediated inhibitory post-synaptic currents in OX neurons

(Chowdhury and Yamanaka, 2016). One explanation could be the co-release of GABA and 5-HT by serotonergic terminals since TPH-producing neurons in the Raphe nuclei have been shown to co-express the GABA rate-limiting enzyme glutamic acid decarboxylase (GAD) (Belin et al., 1983; Day et al., 2004; Fu et al., 2010; Millhorn et al., 1988, 1987; Prouty et al., 2017). However, very few (0.1-0.7%) of serotonergic DR and MR neurons co-express GAD (Fu et al., 2010; Prouty et al., 2017; Stamp and Semba, 1995). This makes less likely that the $GABA_A$-mediated inhibition of OX neurons is due co-release of GABA from PeFR-projecting lwDR/MR serotonergic fibers. A more plausible explanation seems to be the direct activation of local GABAergic interneurons in the PeFR via $5\text{-}HT_{2A}$ receptors, but this remains to be tested. Whatever the explanation may be, the inhibitory effects mediated by the serotonergic neurotransmission onto OX neurons in the PeFR seems to be mediated by 5-HT and GABA.

Even though lesioning of the PeFR-projecting serotonergic neurons did not alter fear acquisition, it did lead to reduced fear extinction when presented with tone only. Although we did not see any significant reduction of 5-HT fibers in the amygdala, we cannot exclude this region from being involved in the alterations of freezing behavior observed in our experiments. This is due the fact that OX neurons project heavily to the amygdala (Peyron et al., 1998a) and play a critical role in the consolidation and extinction of aversive memories, with OX1 antagonist enhancing and OX1 peptide impairing amygdala-based fear extinction in cued fear conditioning (Flores et al., 2014). This role of OX in the context of the mechanisms of persistent fear was further explored by Molosh and colleagues. These authors showed that panic-prone rats submitted to chronic reduction of GABA synthesis in

the PeF had sustained activation of amygdala-projecting PeF pathway and displayed enhanced excitatory synaptic transmission and impaired mGluR2 signaling of principal neurons in the basolateral amygdala (Molosh et al., 2018). Therefore, additional experiments manipulating the orexinergic neurotransmission in the amygdala are needed to confirm its involvement mediating conditioned fear responses in the context of reduced serotonergic neurotransmission in the PeF.

Our data presented here contrast with the view that 5-HT may have only a phasic role in the hypothalamus in the context of innate panic-like behavior as suggested by others (de Bortoli et al., 2013; Nascimento et al., 2014). Our loss-of-function experiments may reflect the fact that ablation of lwDR/MR→PeF serotonergic pathway causes a more profound elimination of 5-HT modulatory effects in the PeFR than does pharmacological inhibition, since this would also alter colocalized neurochemicals. One could raise the possibility that our circuit-targeted ablation may have lesioned other non-serotonergic populations at the Raphe projecting sites. However, the fact that neutral red-positive neurons in the various Raphe nuclei remained unaltered following serotonergic ablation weakens this possibility.

4.4. Conclusions

The past belief that central serotonergic systems are functionally homogeneous in regulating innate panic and conditioned fear was one key barrier. The idea that there are topographically organized subsets of 5-HT neurons, with unique afferents, efferents and functional properties has since become well accepted (Hale et al., 2012). Based on the data presented here and in a series of previous publications, we have identified a novel 5-HT

sub-system that projects to and inhibits a panic generating brain region that also influences conditioned fear responses. These data provide new insights into adaptive panic and fear but also how disruption of this 5-HT system can lead to a vulnerability to panic and conditioned fear responses that are associated with panic attacks, panic disorder (de Beurs et al., 1994; Margraf et al., 1987; Taylor et al., 1986), and highly comorbid phobias (Kessler et al., 2006).

Chapter V: Discussion

5.1. Overall Considerations

Serotonin is heavily implicated in anxiety, fear, and panic, but it seems to have a heterogeneous response depending on the brain region where it is exerting its role. In the amygdala, 5-HT has been largely implicated in promoting anxiety and fear [for review, see (Bauer, 2015; Bocchio et al., 2016; Hindi Attar et al., 2012)] whereas in the PeF only a limited amount of site-specific pharmacological studies support 5-HT's anti-escape role in the context of aversive-promoting environments (Biagioni et al., 2016, 2013; de Bortoli et al., 2013; Nascimento et al., 2014). However, it is hard to determine diffusion rates of site-specific administration of agonists/antagonists, and they most likely fail to recapitulate how physiological DR/MR networks modulate/organize behaviors throughout their down-stream brain targets [for review, see (Hayes and Greenshaw, 2011). A circuit-based approach associated with physiological stimulation parameters is a promising tool to start addressing such questions. Surprisingly, only a few studies investigated the role of specific manipulation of serotonergic network in the amygdala (Ren et al., 2018) and extended amygdala (Marcinkiewcz et al., 2016), and none to our knowledge investigated in the context clinically relevant panicogenic stimuli such as CO_2 challenge.

Our major findings support the hypothesis that serotonergic neurons innervating the BLA and PeF constitute distinct networks concentrated within DRD/DRV and lwDR/MR, respectively. More importantly, selective manipulation of these networks with gain- and loss-of-functions approaches in association with behavioral paradigms assessing anxiety/fear responses and clinically relevant panic provocation induced by CO_2 inhalation

revealed that while DRD/DRV→BLA enhances anxiety- and fear-associated responses, lwDR/MR→PeF dampens panic- and fear-associated responses.

The notion that the amygdala underlies fear emotional states is widely accepted in the neuroscience field and has been extensively studied under conditioned fear paradigms [for review, see (Campese et al., 2016; Izquierdo et al., 2016; Johansen et al., 2011; LeDoux, 2007)]. On the other hand, the involvement of the PeFR on panic-related responses, more specifically the OX-producing neurons, has been proposed by our lab (Johnson et al., 2010) and gained strength over time utilizing mainly pharmacological manipulations [for review, see (Johnson et al., 2014, 2012a). Therefore, before applying our gain- and loss-of-function approaches to question 5-HT's modulation in the PeF, we decided to better characterize the role of PeFR in panic. In order to do that, we used wireless optogenetic approach to selectively stimulate glutamatergic neurons within the PeF.

5.2. Wireless Photostimulation of Glutamatergic and Orexin-Producing Neurons in the Perifornical Hypothalamic Region Elicits Escape Behavior and Intense Cardioexcitation

In our first experiment of Chapter IV, we confirmed that injections of CaMKIIa ChR2 virus led to many eYFP^{+} fibers in the PeFR, and after immunostaining for OXA in control rats, we observed high co-localization between this neuropeptide and eYFP^{+} soma. Optogenetic stimulation of ChR2 group, but not control, led to robust increases in BP and HR that were accompanied by a constant exophthalmos and long periods of freezing interspaced by short vigorous locomotor episodes of escape (running), behaviors typically aversive. These behavioral and physiological responses observed here are similar to those using

chemical (Freitas et al., 2009; Shekhar and DiMicco, 1987; Ullah et al., 2017, 2015), electrical (de Bortoli et al., 2013; Lammers et al., 1988), and optogenetic stimulation (Li et al., 2018) of the PeFR of rats. Particularly in the latter case, selective optogenetic activation of glutamatergic neurons in the PeF/LH that project to the dPAG induced escape from an artificial predator, suggesting the involvement of the PeF in aversive behaviors (Li et al., 2018).

The PeFR was first implicated in the classic "fight-or-flight" behavior almost a century ago by Bard (Bard, 1928) and later by the seminal work of Hess and Brugger (1943). However, classic experiments involving local lesions and non-selective electrical stimulation have also implicated the PeFR as a crucial node responsible for aggressive behavior (Egger and Flynn, 1963; Hutchinson and Renfrew, 1966; Siegel et al., 1999) that induced attack in various species including rat, cat, and monkey (Lammers et al., 1988; Lipp, 1978; Siegel and Pott, 1988). This is likely due the fact that the hypothalamus is a highly complex structure with various anatomical and cellular configurations (Puelles and Rubenstein, 2015; Shimogori et al., 2010), and that the electric current utilized in those studies is a non-selective stimulus that activates not only cell bodies but also fibers of passage. This inevitably limits the conclusions regarding the neuronal groups underlying several behavioral changes observed in those studies.

Contradicting the aggressive behavior reported above, it is noteworthy that optogenetic stimulation of ChR2 group virtually abolished SI time without inducing aggression. This surprisingly selective increase in social anxiety avoidance behavior was accompanied by cFos activation within the PeFR that co-localized with the panic implicated OXA-producing neurons (Johnson et al., 2010). Indeed, recent studies utilizing selective optogenetic

stimulation have shown that the aggressive behavior is particularly associated with the ventromedial hypothalamus (VMH), ventrolateral part (VMHvl) (Lin et al., 2011), more specifically the population expressing the Estrogen receptor 1 (Lee et al., 2014), although other neuronal populations are yet to be tested.

Perhaps the most striking results were not the dramatic place avoidance from the stimulation chamber observed during the training session in the RTPP/A test per se, but the avoidance behavior in the short- and long-term memory sessions. Even though the short-term memory (45 min) avoidance behavior observed here is similar to that described after stimulation of a specific population of $SF1^+$ neurons in the VMH (Kunwar et al., 2015), this is the first time to our knowledge that such long-lasting (24 hours) avoidance behavior is reported post-stimulation of the PeF and adjacent regions. These effects are not only a direct indication of the averseness of glutamatergic stimulation in the PeF, but also indicate the capacity of this region to organize some forms of emotional learning canonically seen in fear conditioning experiments and attributed to the amygdala (LeDoux, 2000; Phelps and LeDoux, 2005). Collectively, optogenetic stimulation of the PeF promotes various orchestrated behavioral and physiological responses that are components of the "fight-or-flight" repertoire, thus reinforcing its role as one of the putative brain regions underlying panic behavior.

5.3. The Amygdala- and Perifornical Hypothalamus-Projecting Serotonergic Systems Originate from Distinct Neuronal Populations in the Dorsal and Median Raphe Nuclei

The following experiments of Chapters III and IV focused on determining which 5-HT systems projected to the BLA and PeF, respectively. Here, we confirmed that injections of a retrograde tracer into the BLA or PeF resulted in many CTB^{+}/TPH^{+} neurons concentrated in the DRD/DRV or lwDR/MR, respectively, with approximately 92-95% of co-localization between CTB^{+} and TPH^{+}, and very few CTB^{+} that were non-TPH cells. It is important to emphasize that the retrograde labelled neurons were restricted to the DR/MR, sparing the surrounding brain regions on the same coronal section. Our anterograde tracers Phal-l into the midline DRD/DRV revealed a high density of fibers in the BLA and a moderate density of fibers within the bed nucleus of the stria terminalis (BNST, **Figure 18a**). These results are consistent with the non-specific retrograde tracer CTB (Hale et al., 2008; Ljubic-Thibal et al., 1999) and with conditional anterograde tracer (Cre-dependent AAV with a fluorescent reporter) that was restricted to serotonergic neurons [SERT-Cre mouse (Gong et al., 2007)] observed in the connectome of the mouse brain in the Allen Brain Atlas (Oh et al., 2014; **Figure 18b**) and by others (Muzerelle et al., 2016). Even though it was already expected, the similar pattern of anterograde tracing confirmed how conserved are the DRD/DRV→BLA and lwDR/MR→PeF projections between these two species, an important issue that should be considered when doing pre-clinical research aiming for translational implications. Collectively, the combination of our anterograde and retrograde tracing studies allowed us to determine not only which site within the BLA and PeF to pursue for our following gain- and loss-of-function experiments, but more importantly it showed how selective were the projections from the serotonergic system to

those sites. We consider this to be of utmost importance since it allowed us to implement the intersectional genetics approach not only to selectively manipulate the serotonergic systems, a matter discussed later, but also to use this same approach as a conditional anterograde tracing to further confirm our neuroanatomical findings with CTB (Chapters III and IV), and Phal-l (Chapter III).

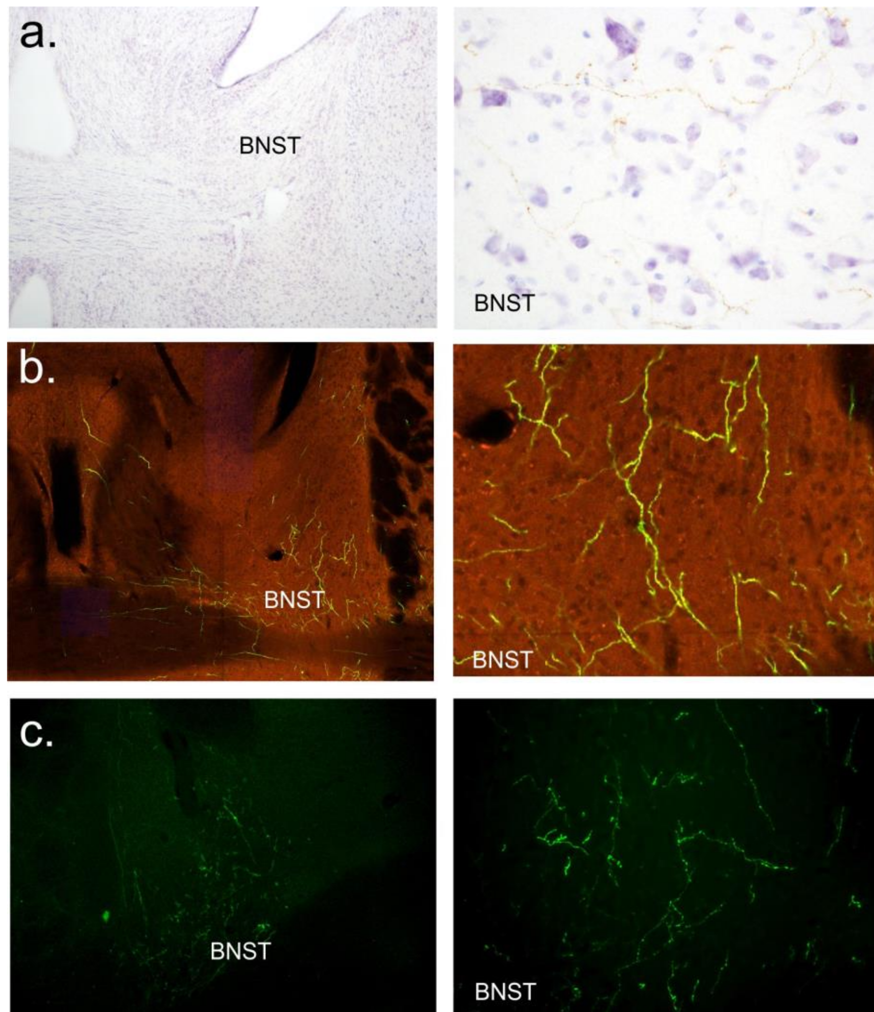


Figure 18 – Basolateral amygdala (BLA)-projecting dorsal (DRD) and ventral (DRV) divisions of the dorsal Raphe nucleus (DR) also send collaterals to the bed nucleus of stria terminalis (BNST). **(a)** Photos are coronal sections of the BNST [orange/brown DAB are Phaseolus vulgaris leucoagglutinin (Phal)-immunoreactive fibers with purple cresyl violet-stained cells of low (left) and high (right) magnification photos from Phal-injected rats from **Figure 10a-c**. **(b)** Photos represent low (left) and high (right) magnification coronal sections of the BNST from a serotonin transporter (SERT)-Cre mouse where a Cre-dependent AAV with a fluorescent reporter was injected into the DR (see **Figure 10d**) and provided by the connectome of the Brain in the Allen Brain Atlas (Oh et al., 2014). **(c)** Photos represent low (left) and high (right) magnification coronal sections of the BNST from a rat with CAV-CMV-Cre injected bilaterally into the BLA and AAV-DIO-EF1a-ChR2-eYFP injected into the midline DR (see **Figure 12**). Image credit for part **(b)**: Allen Institute for Brain Science.

The anatomical findings taken from the intersectional genetic approach were extremely valuable, especially for the PeF-projecting serotonergic system.

DRD/DRV→BLA:ChR2 rats showed highly co-localized $SERT^+/eYFP^+$ fibers in the BLA (Chapter III) that sent collateral inputs to the BNST (**Figure 18** and **Figure 19c**), but not to the PeF (**Figure 19e**). In Chapter IV, lwDR/MR→PeF:ChR2 rats displayed equally co-localized fibers in the PeF and, to our surprise, collaterals in the PAG (**Figure 19d**), but not in the BLA (**Figure 19f**). Therefore, both the BLA and the PeF serve as control sites for each other, as suggested in the findings of previous work (Muzerelle et al., 2016). This has important anatomical implications for the Deakin/Graeff hypothesis elaborated almost 30 year ago (Deakin and Graeff, 1991). At that time, these authors considered the DR as a homogeneous structure, and proposed that serotonergic projections to the amygdala and PAG would respectively facilitate anxiety/fear when processing potential/distal threats, but would restrain unconditioned "fight-or-flight" panic responses to proximal/imminent dangers (Deakin and Graeff, 1991). Therefore, we showed anatomical evidence of two distinct networks projecting to the BLA and, most importantly, our tracing data also suggest that the PeF shares collaterals with the originally proposed panic-restraining circuit projecting to the PAG.

5.4. Loss-of-Function Studies: Lesioning of the Serotonergic Network with Saporin Toxin Dampens Conditioned Fear in the Amygdala and Enhances Conditioned Fear and Innate Panic in the Perifornical Hypothalamic Region

Because 5-HT is heavily implicated in anxiety, fear, and panic, but its role under the circuit-based standpoint is largely unexplored, we sought to determine the role of 5-HT

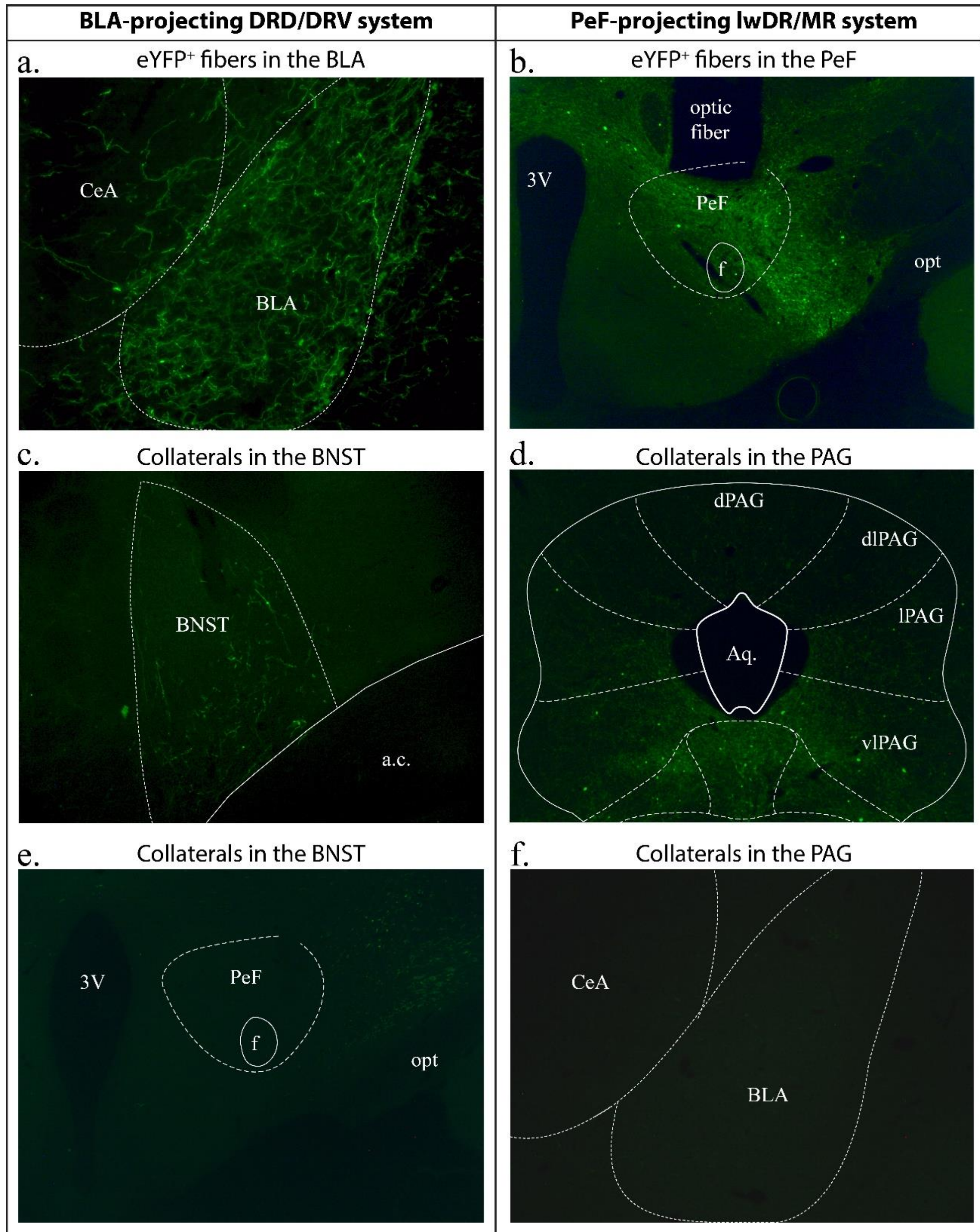


Figure 19 – Different targets of the Basolateral amygdala (BLA)- and perifornical hypothalamus (PeF)-projecting serotonergic networks. **(a)** Amygdala-projecting eYFP$^+$ fibers in the BLA, **(c)** their collaterals in the bed nucleus of stria terminalis (BNST), and **(e)** absence of fibers in the PeF. **(b)** PeF-projecting eYFP$^+$ fibers in the PeF, **(d)** their collaterals in the periaqueductal gray (PAG), and **(f)** absence of fibers in the BLA Abbreviations: 3V third ventricle, a.c. anterior commissure, Aq. cerebral aqueduct, dPAG, dlPAG, lPAG, and

vlPAG are dorsal, dorsolateral, lateral, and ventrolateral PAG, respectively, opt optical tract.

networks in the modulation of anxiety-, fear-, and panic-like behaviors and cardiovascular responses using manipulations of the BLA- and PeF-innervating serotonergic systems. This was first assessed in the loss-of-function studies, in which SERT-SAP was injected into the BLA or PeF. Specific lesions of the serotonergic systems resulted in decreased conditioned fear behavior in the BLA, but increased conditioned fear and innate panic responses in the PeF.

Before discussing the effects on anxiety, fear, and panic, it is important to mention that SERT-SAP-induced lesions produced ~90% and 42% reductions in the density of $SERT^+$ fibers in the BLA and PeF, respectively. These lesions are higher than those reductions in the medulla observed by Nattie et al. (28%) (Nattie et al., 2004). Moreover, injections of SERT-SAP into the BLA and PeF also reduced the total number of TPH^+ neurons almost exclusively in their respective Raphe sources, i.e. DRD/DRV and lwDR/MR, respectively. The only exception was the reduction of a few neurons, yet significant, in the lwDR of the BLA injected SERT-SAP group. Because we injected CTB after SERT-SAP lesioning had already occurred (for experimental timeline, see **Figure 5**), double labelled CTB^+/TPH^+ neuronal counts reflected the previous reductions of single TPH^+ counts observed in the DRV/DRD and lwDR/MR. Accordingly, we also noted that only 5-8% of CTB-expressing cells in the DRD/DRV and lwDR/MR were non-serotonergic. Equally important, animals injected with SERT-SAP in the BLA or PeF did not show alterations in the SERT fiber density of their respective control sites, i.e. PeF and BLA (**Figure 19e** and **f**).

SERT-SAP injection into the BLA did not alter SI behavior but did attenuate conditioned fear response during extinction compared to control group injected with IgG-SAP. Overall, this loss-of-function approach is consistent with our previous work (Johnson et al., 2015b) and previous 5,7-DHT study in which reductions of 5-HT signaling in the amygdala following contextual fear conditioning reduced recall of fear-associated freezing (Izumi et al., 2012). Although we did not see a significant reduction in SI here, we (Johnson et al., 2015b) and others (File et al., 1981) did see significant reductions in SI following 5,7-DHT injections into the amygdala. An explanation to this would be that while we observed lesioning of SERT fibers and their associated cell bodies in the DR/MR here with SERT-SAP treatment, other authors (File et al., 1981; Johnson et al., 2015b) reported only depletion of 5-HT after 5,7-DHT injections (no signs of physical lesioning of SERT fibers nor serotonergic cell bodies).

Partially agreeing with our findings, Baratta and colleagues injected a Cre-dependent Arch-GFP AAV into the midline DR of SERT-Cre mice and observed that optogenetic inhibition of the DR attenuated only stress-enhanced fear (Baratta et al., 2016). The lack of effect on normal cue-induced fear may have been due to use of a reduced shock intensity or species differences. Future studies are needed to understand how exactly lesioning or exciting DRD/DRV 5-HT projections to the BLA is altering electrophysiological activity of BLA projection neurons that control anxiety- and fear-related behavioral responses.

Conversely, selective lesion of the lwDR/MR→PeF serotonergic pathway in Chapter IV increased overall freezing response and delayed extinction during the extinction protocol without altering acquisition of conditioned fear. Although we did not see any significant reduction of SERT^{+} fibers in the amygdala in Chapter IV, we cannot exclude this

region from being involved in the alterations of freezing behavior observed in our experiments. This is due the fact that OX neurons project heavily to the amygdala (Peyron et al., 1998a) and play a critical role in the consolidation and extinction of aversive memories, with OX1 antagonist enhancing and OX1 peptide impairing amygdala-based fear extinction in cued fear conditioning (Dustrude et al., 2018; Flores et al., 2014).

This role of OX in the context of the mechanisms of persistent fear was further explored elsewhere. Molosh and colleagues showed that panic-prone rats submitted to chronic reduction of GABA synthesis in the PeF have sustained activation of amygdala-projecting PeF pathway and display enhanced excitatory synaptic transmission and impaired mGluR2 signaling of principal neurons in the BLA (Molosh et al., 2018). Therefore, additional experiments manipulating the orexinergic neurotransmission in the amygdala are needed to confirm its involvement mediating increased freezing responses and impaired extinction in the context of deficient serotonergic neurotransmission in the PeF.

Interestingly, injection of SERT-SAP into the PeF increased innate anxiety- and panic-associated responses measured by reduced SI time and decreased escape latency in the ETM, respectively, when compared to control IgG-SAP rats. More importantly, our results from the PeF lesioning with SERT-SAP are particularly relevant when compared to rats submitted to the panic-prone model with l-allylglycine leading to chronic GABA disinhibition in the PeF. These rats display similar cardioexcitation and marked increase in innate-anxiety following 7.5% CO_2 inhalation (Fitz et al., 2003) or i.v. infusions of 0.5 M sodium lactate (Johnson and Shekhar, 2006; Shekhar et al., 2006, 1996; Shekhar and Keim, 2000, 1997).

Aside from this panic-prone model, naïve rats exposed to low CO_2 concentrations (7%) display increases only in respiratory activity without mobilizing other components of panic-associated responses (Akilesh et al., 1997). Conversely, higher concentration of CO_2 (20%) elicits not only anxiety- and fear-associated behaviors, but also marked cardiovascular and thermoregulatory responses associated with panic states (Federici et al., 2019; Johnson et al., 2012c, 2011; Spiacci et al., 2018; Ziemann et al., 2009). This latter condition seems to recapitulate the 20% CO_2 panic provocation model used in the clinic to evoke symptoms that match the criteria for PAs in healthy subjects (Forsyth et al., 2000).

Further confirmations of the CO_2 challenge being a suitable option to model PAs in laboratory animals can be observed elsewhere. Spiacci and colleagues showed that treatments with Fluoxetine and Alprazolam, two clinically relevant panicolytic drugs utilized to treat patients with PD, are able to attenuate escape responses elicited by 20% CO_2 inhalation (Spiacci et al., 2018).

Even though these studies collectively show that the CO_2 challenge can be used to model PAs in animals, none of them manipulated the serotonergic network innervating the PeF in the context of CO_2-induced panic provocation. We utilized the same CO_2 challenge paradigms (7 and 20%) in order to address this issue. Interestingly, lesioning of the lwDR/MR→PeF serotonergic network enhanced general motor activity and increased BP in the CO_2 challenge, facilitating panic-like responses.

These effects are in agreement with the potentiation of the defensive responses elicited by chemical stimulation of the PeF post local lesioning of 5-HT fibers with local injections of 5,7- DHT (Gołebiewski and Romaniuk, 1985). Because it has been previously

shown that OX-expressing neurons in the PeF are directly inhibited by 5-HT via post-synaptic 5-HT_{1A} receptors (Chowdhury and Yamanaka, 2016; Muraki et al., 2004; Saito et al., 2018), our results from SERT-SAP injections can be explained by direct effects of reduced inhibitory tone due loss of serotonergic innervation onto OX neurons in the PeF. Alternatively, OX neurons also express $GABA_A$ receptor subunits and are excited by bicuculline methiodide and inhibited by muscimol, antagonist and agonist of $GABA_A$ receptor, respectively (Eggermann et al., 2003). One explanation could be the co-release of GABA and 5-HT by serotonergic terminals since TPH-producing neurons in the Raphe nuclei have been shown to co-express GAD (Belin et al., 1983; Day et al., 2004; Fu et al., 2010; Millhorn et al., 1988, 1987; Prouty et al., 2017). However, very few (0.1-0.7%) of serotonergic DR and MR neurons co-express this enzyme required for GABA synthesis (Fu et al., 2010; Prouty et al., 2017; Stamp and Semba, 1995), which weakens the assumption that $GABA_A$-mediated inhibition of OX neurons are due co-release of GABA from PeFR-projecting lwDR/MR serotonergic fibers. A more plausible explanation seems to be the direct activation of local GABAergic interneurons in the PeFR via 5-HT_{2A} receptors, a matter yet to be investigate.

Another alternative one could consider is the participation of glutamate signaling. Even though the co-release of 5-HT and glutamate has been reported for various Raphe nuclei (Amilhon et al., 2010; Hioki et al., 2010), only 5% of the TPH2-producing neurons in the lwDR co-localize with vesicular glutamate transporter 3 (Hioki et al., 2010). This helps explain the lack of direct effects of glutamate neurotransmission in OR neurons after optogenetic stimulation of serotonergic terminals in PeF slices (Chowdhury and Yamanaka, 2016), making this a less likely option in this region.

Our data presented here contrast with the view that 5-HT may have only a phasic role in the hypothalamus in the context of innate panic-like behavior as suggested by others (de Bortoli et al., 2013; Nascimento et al., 2014). Our loss-of-function experiments may reflect the fact that ablation of lwDR/MR→PeF serotonergic pathway causes sustained decreases of 5-HT in the PeF when compared with the transient inhibition obtained with pharmacological approach. Therefore, our loss-of-function approach reflects more closely pathological states in which patients diagnosed with PD were suggested to have reduced serotonergic neurotransmission [for review, see (Graeff, 2017)]. One could raise the possibility that our circuit-targeted ablation may have unspecific effects at non-serotonergic neurons in the Raphe projecting sites. However, the fact that SERT-SAP did not change total number or neurons assessed by neutral red staining in the various Raphe nuclei weakens this possibility.

5.5. Gain-of-Function Studies: Photostimulation of Serotonergic Network Enhances Anxiety and Conditioned Fear in the Amygdala and Dampens Conditioned Fear and Panic in the Perifornical Hypothalamic Region

The foregoing loss-of-function experiments raised the question whether the decreased conditioned fear and facilitation of innate anxiety- and panic-like responses could be reverted if we used a circuit-based approach that enhanced 5-HT release in the BLA or PeF, respectively. To address this matter, we took advantage of the highly selective serotonergic projections (92-95%) from the DRD/DRV to the BLA or lwDR/MR to the PeF observed in our previous anatomical experiments and utilized a combination of the retrogradely trafficked Cre-expressing CAV (Soudais et al., 2001) injected either in the BLA or

in the PeF and a Cre-dependent ChR2 in the DR/MR. As discussed above, the eYFP expression in the Raphe nuclei confirmed the anatomy of our retrograde tracing results and data in the literature (Ljubic-Thibal et al., 1999; Muzerelle et al., 2016), with the BLA and PeF injected animals co-expressing TPH and eYFP in DRD/DRV and lwDR/MR, respectively.

Surprisingly, photostimulation of BLA-projecting DRD/DRV serotonergic cell bodies per se did not produce any behavioral nor cardiovascular responses in resting rats (no aversive stimuli were given) habituated to the stimulation box (**Figure 20**). It has been shown that electrical or chemical stimulation of the basolateral or central amygdala of cats, rabbits, or rats can produce behavioral and physiological changes that resembles a state of fear, such as cessation of ongoing behavior, increase in BP, HR, and plasma levels of corticosterone (Applegate et al., 1983; Dunn and Whitener, 1986; Feldman et al., 1982; Harper et al., 1984; Iwata et al., 1987). Accordingly, in humans, even though not all subjects reported subjective emotional responses during amygdala stimulation trials (Meletti et al., 2006), stimulation to the amygdala can induce an array of emotional experiences ranging from positive (e.g. elation) to negative emotional (e.g., fear) (Bijanki et al., 2014; Inman et al., 2018; Lanteaume et al., 2007; Meletti et al., 2006; Smith et al., 2006). The negative emotional responses are more frequently observed at higher amplitudes of stimulation, and are commonly followed by increased skin conductance and increase in HR (Inman et al., 2018), responses typically observed in fear-promoting situations.

However, we have to take in consideration that while electrical stimulation directly activates neurons and fibers of passage, chemical stimulation is mediated by glutamate neurotransmission, the main excitatory neurotransmitter in the brain. Serotonin, on the

other hand, serves as a neuromodulator and particularly in the amygdala it is suggested to regulate the excitability of BLA principal neurons directly via 5-HT_{2C} receptors (Greenwood et al., 2012; Li et al., 2003) and indirectly via local GABAergic interneurons

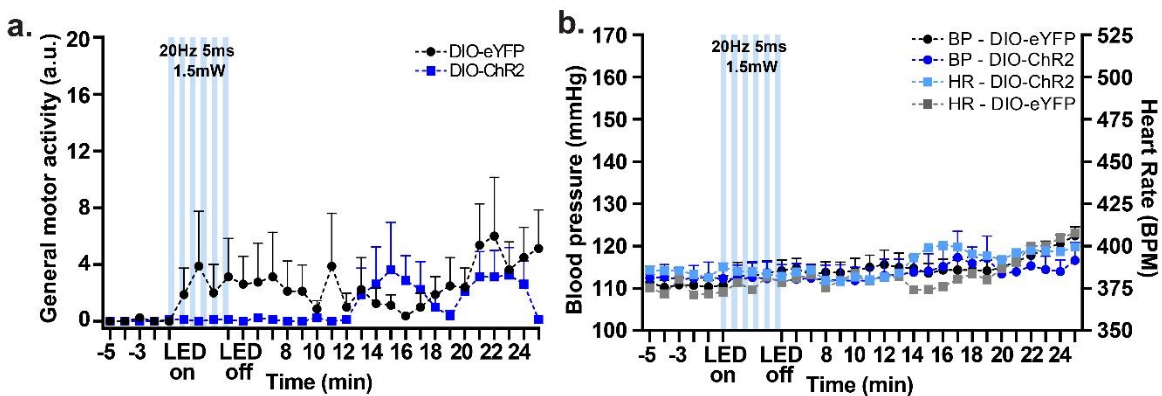


Figure 20 – Optogenetic stimulation of basolateral amygdala (BLA)-projecting DRD/DRV serotonergic cell bodies. Photostimulation (20 Hz, 5 ms, 1.5 mW, LED on-LED off, 5 min) of DRD/DRV cell bodies did not produce **(a)** general motor activity (circle and square signs), **(b)** blood pressure (BP, circle signs, left y axis), nor heart rate (HR, square signs, right y axis) in freely moving rats.

expressing 5-HT_{2A} receptors (Jiang et al., 2009; McDonald and Mascagni, 2007; Rainnie, 1999; Yamamoto et al., 2012). Therefore, in the absence of aversive stimuli that normally trigger amygdala activity, serotonergic neurotransmission per se may very well have little, if any, effects on BLA firing rate. The lack of behavioral and cardiovascular responses observed here are an indication of this.

Serotonin had a different effect in the presence of an aversive stimulus. Even though photostimulation of DRD/DRV→BLA:ChR2, but not eYFP control, did not alter fear acquisition nor consolidation, it did increase freezing in the initial tone presentation during recall/extinction session. The stimulation also led to anxiety-associated reduction in SI time in the open field. Accordingly, we then verified that optogenetic stimulation also increased cellular cFos response within serotonergic neurons in the DRV and DRD and within the cells in the BLA. In agreement with our results, Baratta and colleagues injected

a Cre-dependent Arch-GFP AAV into the midline DR of SERT-Cre mice and observed that optogenetic inhibition of the DR attenuated conditioned fear responses only in stressed animals (Baratta et al., 2016). The lack of effect on normal cue-induced fear may have been due to use of a reduced shock intensity or species differences.

Although we observed significant collaterals in the BNST, we did not see a significant increase in cFos in this region. Even though this a negative finding, it is noteworthy that optogenetic excitation of ChR2 expressing 5-HT terminals in the BNST of SERT-Cre mice enhanced anxiety-related behaviors and conditioned fear behaviors (Marcinkiewcz et al., 2016). Based on our anatomical experiments, the source of some of the serotonergic inputs to the BNST are shared collaterals of BLA-projecting neurons within the midline DR.

In regards to what is known about 5-HT effects in the BLA there are two factors to consider: 5-HT induces both excitatory and inhibitory actions depending on the receptor subtype and 5-HT receptor subtypes are expressed on both GABAergic interneurons and glutamatergic projection neurons (McDonald and Mascagni, 2007). Glutamatergic principal neurons that enhance anxiety and conditioned fear responses have high expression of excitatory 5-HT_{2C} receptors in the dorsal part of the BLA [i.e., LA; (Greenwood et al., 2012; Li et al., 2003)] and 5-HT excites these neurons via the 5-HT_{2C} receptor (Yamamoto et al., 2014). Additionally, acute injections of 5-HT, an SSRI, or a 5-HT_{2C} receptor agonist into the BLA induce anxiogenic responses (Vicente and Zangrossi, 2012). Yet, application of 5-HT in the BLA region initially produces inhibitory responses by depolarizing GABAergic interneurons (Bocchio et al., 2015; Rainnie, 1999) which have high expression of

excitatory 5-HT_{2A} receptors (McDonald and Mascagni, 2007) and play a role in the depolarization (Bocchio et al., 2015). However, previous studies show that stress- and fear-related conditions can produce prolonged release of 5-HT that may lead to loss of local inhibition (Amat et al., 1998; Zanoveli et al., 2009), and there is evidence that this can potentially reduce local GABA inhibition and produce excitation of glutamatergic neurons. For instance, stress exposure can induce downregulation of 5-HT_{2A} receptor expression in the BLA and reduce 5-HT's effects on local GABAergic tone (Jiang et al., 2009). This could lead to excitation of glutamatergic neurons, which play a critical role in enhancing fear conditioning. This hypothesis is supported by previously mentioned studies where initial treatment of rats with SSRIs increases extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001), enhances initial fear-related freezing response in rodents (Ravinder et al., 2013), and enhances conditioned fear in humans (Grillon et al., 2007).

Overall, the aforementioned data support the hypothesis that serotonergic neurons in the DRD/DRV that project to the BLA enhance anxiety-like behavioral and conditioned fear response.

In Chapter IV, photostimulation of lwDR/MR→PeF fibers induced arousal in resting rats habituated to the stimulation box. Even though there was a mild, yet significant, increase in the cardiovascular parameters during optogenetic stimulation, it is noteworthy that the behavior repertoire was qualitatively different from those observed in our first experiment with optogenetic excitation of glutamatergic cell bodies in the PeF (see section 5.2). Here, rats displayed investigation-driven behaviors, such as increased exploration, accompanied by sniffing and occasional rearing, and we cannot discard the possibility that these factors may have contributed to the cardioexcitation. In agreement with these results,

5-HT release in the hypothalamus has been linked to the control of wakefulness responses in different stances (Houdouin et al., 1991; Imeri et al., 1994; Yuki C. Saito et al., 2018).

Interestingly, we observed that photostimulation conditions that initially evoked arousal/increased exploration at neutral environments were also associated with 1) increased spatial exploration of the inherently aversive open arm in the EPM, and 2) augmented social exploration/interaction with or without anxiogenic/panicogenic stimuli in the SI test. Regarding the EPM, ChR2 excitation increased open arm exploration even after the light stimulation ceased, suggesting a sustained anti-aversive effect of 5-HT in the PeF. This can be explained when we take into account that 5-HT neurotransmission has been reported to be predominantly non-junctional, i.e. when the localization of the ligand's receptors lack direct synaptic contact as observed for several brain regions (Descarries et al., 1990; Umbriaco et al., 1995). Even though anatomical confirmations have not been yet established for the PeF, electrophysiological data suggest that 5-HT can use volume transmission as a mode of communication in the PeF as there was a significant delay (approximately 7 seconds) from the time between 5-HT terminals activation and recorded inhibitory post-synaptic currents in OX neurons (Chowdhury and Yamanaka, 2016).

With respect to SI, activation of ChR2 fibers in the PeF increased SI time during stimulation session and blunted the anxiogenic effects of bright light challenge. Although photostimulation of lwDR/MR→PeF fibers post-CO_2 challenge did not prevent a significant reduction in SI time when compared to the same group during the stimulation session, serotonergic network activation still countered the aversive effects of CO_2 inhalation since SI time of ChR2 animals was higher than control group within the same session. Collectively, these tests are strong evidences of anxiolytic properties mediated by

lwDR/MR→PeF optogenetic stimulation as these paradigms are validated models for testing anxiety-like behavior in rodents (File, 1980; Pellow et al., 1985; Pellow and File, 1986).

As mentioned in the section 5.4, panic prone and naïve rats and PD and healthy subjects seem to have similar responses to mild (7%) and high (20%) CO_2 inhalations. Despite this particularly relevant similarity, none of the previous studies focused on investigating the specific effects of 5-HT neurotransmission in the PeF in the context of CO_2-induced panic provocation. In order to address this issue, we utilized the same CO_2 challenge paradigms (7 and 20%) coupled to photostimulation of 5-HT fibers of the lwDR/MR→PeF circuit. Remarkably, excitation of 5-HT fibers was able to attenuate the BP increase induced by both CO_2 challenges used here. More importantly, the BP response from ChR2 group submitted to 20% CO_2 was similar to the BP levels of rats from control group exposed to 7% CO_2. Additionally, our post-mortem analysis showed that photostimulation of the PeF-projecting lwDR/MR fibers prior to euthanasia was able to decrease cell activity of OX A-expressing neurons assessed by cFos activation. This is particularly interesting when taking into consideration previous studies from our lab showing the importance of the orexinergic neurotransmission and OX1 receptor underlying panic-like responses evoked by CO_2 challenges (Bonaventure et al., 2017; Johnson et al., 2015a, 2012c).

As explored previously, 5-HT has inhibitory effects onto OX-producing neurons in the PeF (Chowdhury and Yamanaka, 2016; Muraki et al., 2004), but to our knowledge, this study is the first to unveil causal relationship regarding which neuronal populations in the Raphe nuclei are implicated in the attenuation of panic-associated responses utilizing a circuit-based approach. Equally important, these findings are also extremely relevant to update the influential Deakin/Graeff hypothesis. Initially overlooked by these authors, here

we identify specific serotonergic projections from lwDR/MR to the PeF and provide evidence that direct activation of such circuit confers anti-panic properties in both ethologically and clinically relevant models of panic. Along those lines, the identification of specific circuits controlling anxiety, fear, and panic responses is a fundamental step towards the development of more effective therapies for psychiatric conditions such as anxiety and trauma-based disorders (e.g. PD and PTSD, respectively).

Lastly, because previous photostimulation of glutamatergic cell body in the PeF produced a remarkable avoidance behavior in RTPP/A test that extended up to 24 hours post-stimulation, we questioned what would be the behavioral effects of optogenetic stimulation of lwDR/MR→PeF pathway using the same model. To our surprise, photostimulation of this pathway led to a marked place preference behavior during training session that persisted not only for 45 min (short-term), but also lasted 24 hours thereafter (long-term memory session). Since our optical fibers were indisputably located above the PeF, the most parsimonious explanation would be that these reward-like behaviors were due activation of PeF-projecting lwDR/MR neurotransmission.

However, because there were additional fibers in the LH, we cannot exclude the possibility that our lwDR/MR→PeF terminal activation was 1) stimulating fibers of passage that innervated the LH and also expressed ChR2; and/or 2) stimulating local PeF fibers that collateralized to the LH. Indeed, the LH has long been proposed as a brain area that produces self-stimulation and reward-like behaviors with electrical stimulation (Olds, 1958, 1956; Olds and Milner, 1954). Moreover, recent optogenetic studies demonstrated that activation of DR 5-HT neurons promotes reward (Fonseca et al., 2015; Miyazaki et

al., 2014), and these effects were suggested to be mediated by projections to ventral tegmental area (Liu et al., 2014; Wang et al., 2019) and nucleus accumbens (Liu et al., 2014). In the LH, pharmacological studies often provided conflicting results regarding the role of 5-HT either promoting or suppressing reward-related behaviors [for review, see (Luo et al., 2015)]. Such contradiction can be explained by the fact that it is hard to determine diffusion rates of intracranial administration of specific receptor agonist or antagonist. Moreover, such drug deliveries inevitably miss the global mechanism of how physiological DR neurotransmission modulates/organizes behaviors throughout their downstream brain targets [for review, see (Hayes and Greenshaw, 2011). A circuit-based approach associated with physiological stimulation parameters seems to be the neuroscientist's latest and best tool to start tackling such questions. Even though we cannot answer whether the place preference observed here was mediated by lwDR/MR neurotransmission in the PeF or LH, or the combination of both, this certainly poses an interesting venue that deserves to be addressed in future studies.

Chapter VI: Concluding Remarks and Future Directions

In this dissertation, our retrograde tracing results demonstrated that anatomically different DR sub-systems and MR innervate the BLA and PeFR in a distinct manner. While the BLA-projecting neurons were localized in the DRD/DRV, the PeFR-projecting serotonergic neurons were concentrated in the lwDR and MR. In both cases, the projections were almost exclusively serotonergic.

Our behavioral and physiological analyses in the gain- and loss-of-function studies reflected the aforementioned anatomical pattern, and in most cases manipulation of these BLA- and PeFR-projecting serotonergic sub-systems resulted in distinct, even opposing, functions. While lesioning of the BLA-innervating 5-HT network facilitated the extinction of conditioned fear behavior, the PeFR counterpart not only did the opposite to conditioned fear responses, but also increased social anxiety avoidance, panic-associated escape, and enhanced cardioexcitation during suffocation-related CO_2 challenges. In the gain-of-function studies, selective activation of BLA-projecting 5-HT networks with wireless LED impaired the extinction of conditioned fear behavior and enhanced social anxiety avoidance. Conversely, excitation of lwDR/MR $ChR2^+$ terminals in the PeFR decreased anxiety associated behaviors, induced conditioned place preference, and attenuated CO_2-induced panic (e.g., flight/escape behaviors and cardioexcitation).

Collectively, the results provided here support previous findings that the DR should no longer be viewed as a homogeneous cluster of cells (Commons, 2016, 2015; Hale and Lowry, 2011; Ren et al., 2018), but rather as sub-systems with unique projection patterns that are capable of controlling distinct, and even opposing, functions, such as conditioned

fear responses. While the results presented in this dissertation provide new insights regarding the neural circuits within the DR and MR underlying the modulation of anxiety, fear, and panic, they also raise important questions about more selective pharmacological alternatives for treating the different anxiety and trauma-based disorders.

Currently, SSRIs are the first line of pharmacological treatment for anxiety and trauma-based disorders, such as PD and PTSD, respectively [for review, see (Koen and Stein, 2011)]. The anxiolytic effects of SSRI therapy occur 2-3 weeks following daily treatments and there is evidence that they actually increase anxiety initially (Masand and Gupta, 1999; Spigset, 1999; Teicher et al., 1990). Based on our results, this initial increase in anxiety could be explained by the fact that SSRIs do not distinguish between the different serotonergic sub-systems in the Raphe nuclei, ultimately increasing 5-HT release in various regions, including the BLA. A way to circumvent these unwanted side effects would be the development of new, more selective, drugs capable of targeting specific 5-HT sub-systems while sparing others.

We have provided evidence that help understand the complexity of the DR and MR serotonin system. Although challenging, such effort can aid the development of more effective pharmacotherapies for brain disorders involving anxiety, fear, and panic. However, follow up experiments are still needed to meet this challenging task. For instance, even though the DR and MR networks manipulated here were highly selective for 5-HT (92-95%), one could argue what effects did these remnant fibers have on the behavioral and physiological parameters we tested. This question can be addressed with the aid of Cre-expressing lines restricted to TPH and SERT neuronal populations in association with the intersectional genetics approach to virtually exclude off-target expression of ChR2. *In vitro*

experiments involving selective manipulation of the BLA and PeF 5-HT networks and electrophysiology dissecting the contribution of specific 5-HT receptors and possible co-released neurotransmitters would also help advance the knowledge about the mechanisms underlying local cell activation/inhibition. Lastly, because the Raphe system has distinct molecular signatures (Calizo et al., 2011; Fernandez et al., 2015; Gaspar and Lillesaar, 2012; Kiyasova et al., 2011), it would be of utmost importance to determine eventual idiosyncrasies of the BLA- and PeF-projecting serotonergic neurons. One way to achieve this is through specialized flow cytometry (fluorescence-activated cell sorting, or FACS), which provides a method for sorting a heterogeneous mixture of cells, one at a time, based upon the specific light scattering and fluorescent characteristics of each cell. As the field of individualized pharmacotherapy based on molecular profiling advances, identifying molecular signatures of the aforementioned 5-HT systems through FACS seems a viable option to provide new alternatives for drug development in the field of anxiety and trauma-based disorders.

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Curriculum Vitae

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Education

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M.Sc.	Program in Physiological Sciences Graduate Program in Physiological Sciences Federal University of Espirito Santo Vitoria, ES, Brazil Advisor: Luiz Carlos Schenberg, Ph.D.
B.S.	Biological Sciences Federal University of Espirito Santo Vitoria, ES, Brazil Advisor: Valeria Fagundes, Ph.D.

Honors and Awards

2019	Selected for Oral Presentation at Stark Summer Science Symposium
2017	Larry Kays Fellowship

2015	Stark Fellowship
2015	F1000Prime recommendation
2013	Monetary Award for the Best Graduate Research at the VIII Regional Meeting of the Brazilian Federation of Experimental Biology Societies
2008	Monetary Award for the Best Undergraduate Research at the LIV Brazilian Congress of Genetics
2006-2008	Recipient for two consecutive years of the National Council for Research and Development (CNPq) Fellowship for undergraduate research

Manuscripts

In preparation	Identification of a Novel Perifornical Hypothalamic Projecting Serotonergic System that Inhibits Innate Panic and Conditioned Fear Responses. **Cristian S. Bernabe,** Izabela F. Caliman, Erik Dustrude, Andrei I. Molosh, Anantha Shekhar, Philip Johnson.
Submitted	Using loss of and gain of function approaches to identify amygdala projecting serotonergic neurons in the dorsal Raphe nucleus that enhance anxiety and fear conditioned behaviors. **Cristian S. Bernabe**, Izabela Caliman, William A. Truitt, Andrei Molosh, Anantha Shekhar, Philip L. Johnson. (Submitted to *Journal of Psychopharmacology*).

Submitted	Chemogenetic excitation of the orexin hypothalamic system mobilizes a coordinated panic response and efferent panic network. Philip L. Johnson, **Cristian S. Bernabe**, Izabela Caliman, Lauren M. Federici, Andrei Molosh, Aline Rezende Ribeiro Abreu, Stephanie D. Fitz, Brock Shireman, Pascal Bonaventure, William Truitt, Seema Bhatnagar, Brian Samuels, Anantha Shekhar. (Submitted to *Journal of Psychopharmacology*)
2019	Assessment of fear and anxiety associated behaviors, physiology and neural circuits in rats with reduced serotonin transporter (SERT) levels. Philip L. Johnson, Andrei I. Molosh, Lauren M. Federici, **Cristian S. Bernabe**, David Haggerty, Stephanie D. Fitz, Eugene Nalivaiko, William Truitt, Anantha Shekhar. *Translational Psychiatry*.
2018	Orexin depolarizes central amygdala neurons via orexin receptor 1, phospholipase c and sodium-calcium exchanger and modulates conditioned fear. Erik T. Dustrude, Izabela F. Caliman, **Cristian S. Bernabe**, Pascal Bonaventure, Seema Bhatnagar, Philip L. Johnson, Andrei I. Molosh, Anantha Shekhar. *Frontiers in Neuroscience*
2017	Long-term treatment with nandrolone decanoate impairs mesenteric vascular relaxation in both sedentary and exercised female rats. Izabela F. Caliman, **Cristian S. Bernabe**, Antônio F. de Melo, Girlândia A. Brasil, Andrews M. do Nascimento, Ewelyne M. de Lima,

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2015 Pharmacological depletion of serotonin in the basolateral amygdala complex reduces anxiety and disrupts fear conditioning. Philip L. Johnson, Andrei Molosh, Stephanie D. Fitz, Dave Arendt, Gerald A. Deehan, Lauren M. Federici, **Cristian S. Bernabe**, Eric A. Engleman, Zachary A. Rodd, Christopher A. Lowry, and Anantha Shekhar. *Pharmacology, Biochemistry and Behavior*.

2015 In a rat model of panic, corticotrophin responses to dorsal periaqueductal gray stimulation depend on physical exertion. Rubia de S. Armini, **Cristian S. Bernabe**, Caroline A. Rosa, Carlos A. Siller, Fagna G. Schimitel, Sergio Tufik, Donald F. Klein, Luiz C. Schenberg. *Psychoneuroendocrinology*. (F1000Prime recommendation).

2014 Translational approach to studying panic disorder in rats: hits and misses. Luiz Carlos Schenberg, Fagna G. Schimitel, Rubia de S. Armini, **Cristian S. Bernabe**, Caroline A. Rosa, Sergio Tufik, Claudia

J. Müller, Jeyce W. Quintino-dos-Santos. *Neuroscience Biobehavioral Reviews.*

2014 Social learning deficits and amygdala network disruptions in neurofibromatosis type 1 and their rescue by p21-activated kinase 1 deletion. Andrei I. Molosh, Philip L. Johnson, John P. Spence, David Arendt, Lauren M. Federici, **Cristian S. Bernabe**, Steven P. Janasik, Zaneer M. Segu, Rajesh Khanna, Chirayu Goswami, Weiguo Zhu, Su-Jung Park, Lang Li, Yehia S. Mechref, D. Wade Clapp, and Anantha Shekhar. *Nature Neuroscience*.

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