Selecting Anxiety: The Central Extended Amygdala as an Arbiter of Emotion-Relevant Responses

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Introduction

Anxiety disorders are among the most common psychiatric diagnoses, affecting roughly one in three people over the lifespan (Kessler et al., 2012; US Burden of Disease Collaborators, 2018). These disorders are linked to serious adverse outcomes and are a leading cause of disability worldwide (Baxter et al., 2013; Beddington et al., 2008; Vos et al., 2016). Transdiagnostic features like inhibited temperament and dispositional negativity are risk factors for the development of these disorders (Moser et al., 2015; Shackman et al., 2016). At their core, these features reflect an impaired ability to select adaptive emotion-relevant responses, which can manifest as maladaptive behaviors and worsen anxiety (Shackman et al., 2016). In this chapter, we review translational evidence suggesting that the central extended amygdala (EAc) can promote maladaptive responses when it becomes dysregulated (Alheid, 2003; Fox et al., 2015; Shackman & Fox, 2016). The EAc is best known for its role in defensive responding, and we review evidence of its critical involvement in threat processing. However, rodent findings reveal that the EAc is also deeply involved in promoting a range of appetitive and consummatory behaviors. These exciting findings suggest that the function of the EAc is not simply to make us feel anxious or afraid, but rather to select between competing emotion-relevant responses that optimize fitness across a variety of survival-relevant contexts. Here, we outline how EAc dysregulation can dispositionally bias an individual toward the selection of inhibited behaviors, in contextually inappropriate situations. These insights form a translational framework for investigating the mechanisms our brains use to select adaptive emotion-relevant responses, and how alterations of those mechanisms can lead to anxious pathology.

Temperament Reflects Our Emotion-Relevant Response Patterns

Imagine that you're out for a picnic with two friends. You meet at a park, walk to a hilltop, and begin setting up your meal. Just then, you hear thunder in the distance. You look up and see that low, black clouds are headed your way. You check the weather forecast and let your friends know that there's a 50-percent chance of a severe thunderstorm. You and your companions know that thunderstorms can be dangerous: Apart from their potential to spoil your picnic, the lightning strikes they unleash can cause serious injury or even death. Yet for many of us, it's probably easy to imagine a specific friend who might opt to get indoors as quickly as possible, and another who would carry on, unaffected by the ominous skies. How could this be? One explanation is that we each have our own individual *temperament*—a set of unique, partially heritable idiosyncrasies that add up to who we are. Some of us are inherently circumspect. Others are gratuitous thrill seekers. Some of us prefer our picnics with dry clothes and dry Rieslings. Others like piña coladas and getting caught in the rain.

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An individual's tendency to express some emotions more frequently or intensely than others is a defining feature of temperament (Fox, 1998; Rothbart, Sheese, Rueda, & Posner, 2011). Individuals express their temperament as dispositional tendencies toward *emotion-relevant response patterns*, such as those of our picnic-goers described above. Of course, variation in temperament colors our social experience and makes life interesting. An extremely anxious or inhibited temperament, however, is also associated with an increased risk for the development of mental health disorders (Bar-Haim et al., 2009; Biederman et al., 2001; Clauss & Blackford, 2012; Reniers et al., 2016; Schwartz et al., 1999, 2003; Essex et al., 2010). An extremely anxious or inhibited temperament is characterized by shyness and reticence toward novel objects and situations (Kagan, 1997; Kagan et al., 1988), as well as patterns of especially risk-averse behavior. These individuals tend to behave as though threats are more likely, serious, or imminent than they actually are, and they're at heightened risk of developing anxiety disorders (Biederman et al., 2001; Essex et al., 2010; Schwartz et al., 1999). As we will discuss throughout this chapter, the precise brain circuits and mechanisms that underlie this heightened risk are beginning to come into focus, thanks largely to advances in translational neuroscience.

Anxiety Disorders are Common, Debilitating, and Self-Reinforcing

Anxiety disorders constitute the most prevalent clinical psychiatric conditions and are among the most common and debilitating of these conditions (Baxter et al., 2013; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; US Burden of Disease Collaborators, 2018; Vos et al., 2016). Roughly one in three people will experience at least one anxiety or depressive disorder over the course of their lifetime (Baxter et al., 2013; GBD 2016; Disease and Injury Incidence and Prevalence Collaborators, 2017; Vos et al., 2016). Apart from imposing multibillion-dollar cost burdens on national healthcare economies (Beddington et al., 2008), these disorders are associated with considerable suffering; are frequently comorbid with other psychiatric diagnoses (Gorman, 1996; Kaufman & Charney, 2000; Kessler et al., 2005; Sareen, 2014); and are associated with a range of adverse outcomes, including job insecurity (Himle et al., 2014; Lecrubier et al., 2000), homelessness (Levorato & Bocci, 2017; North et al., 1998), alcohol and substance abuse (Edwards et al., 2012; Swendsen et al., 2010), and suicide (Nepon et al., 2010). Understanding the factors that contribute to elevated risk for anxiety disorders is critical to early intervention and promises to inform new behavioral and pharmacological treatment approaches.

Dispositional negativity and extremely anxious or inhibited temperaments are associated with negative emotional states that are common to anxiety disorders. These states are accompanied by persistent worry about uncertain future events, which can be overwhelming even when the threats they pose are unlikely, minor, or remote (Grupe & Nitschke, 2013; Shackman et al., 2016). Individuals who suffer from anxiety disorders are often highly reactive to acute stressors and uncertain anticipation (Grupe & Nitschke, 2013), and it has been proposed that their increased likelihood of encountering these stressors reinforces and exacerbates their anxious pathology (Shackman et al., 2016; *Figure 1*). For example, these individuals are often inclined toward social withdrawal and tend to behave in ways that lead to social rejection, conflict, and other adverse outcomes (e.g., divorce, unemployment, etc.) that promote even deeper withdrawal and amplify their negative emotional states (Shackman et al., 2016).

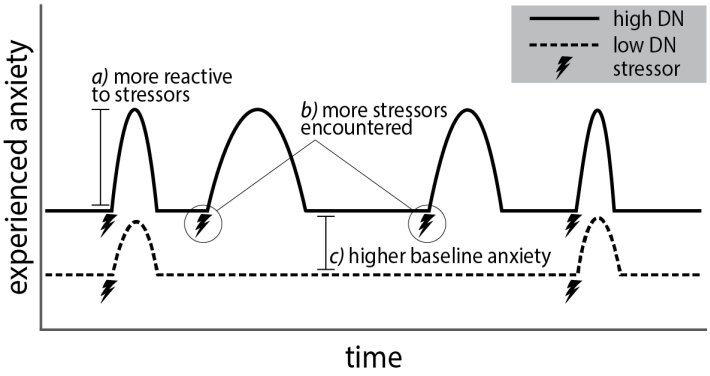

Figure 1: A theoretical framework showing how higher levels of dispositional negativity (DN) put individuals at increased risk of experiencing momentary anxiety through three contributing pathways: first, these individuals tend to be more reactive to stressors (a); second, they tend to encounter stressors more frequently (b); and third, they experience higher baseline anxiety in the absence of stressors (c) compared to low-DN peers. Adapted from Shackman et al., 2016.

The Cycle of Extreme Anxiety and Maladaptive Responses

Anxiety is an adaptive response that focuses attention and primes the body for action when threats and challenges loom (Fox et al., 2015; Lang & McTeague, 2009; Shackman & Fox, 2016). Contrary to its much-maligned reputation, anxiety is a good and necessary part of our everyday experience that promotes adaptive behavior. Each of us experiences anxiety to varying degrees across an expanse of contexts. Adaptive anxiety's importance is perhaps most succinctly illustrated by an ecological example. When a gazelle grazes, it accepts an increased risk of predation to secure food rewards. Adaptive anxiety impels the gazelle to periodically stop eating, look up, and scan for predators (Blanchard et al., 2011; Blanchard & Blanchard, 2008; Cooper & Blumstein, 2015). Without anxiety, the gazelle would fail to detect environmental threats and quickly find itself on a predator's menu. If the gazelle is too anxious, on the other hand, it might spend excessive time scanning for predators and fleeing from ambiguous threat cues. In this case, the animal could find itself in desperation mode and be forced into gratuitously risky behavior as malnourishment sets in. Natural selection would penalize either of these extreme phenotypes, and so nature "calibrates" anxiety somewhere in-between, promoting a survival-optimizing balance of risk-taking and reward-seeking behavior.

Human anxiety serves a similar purpose: When it is *adaptive*, it guides our behavior and primes our bodies to take the right risks at the right time, allowing us to avoid dangers and seize opportunities. But when anxiety is extreme, prolonged, frequent, or contextually inappropriate, it can become debilitating and promote maladaptive behavior. Like a gazelle that spends too much time scanning for predators to secure a meal, people who experience anxiety disorders tend to fixate so intensely on the possibility of adverse future events that they forgo valuable rewards to hedge against threats that are unlikely, minor, or remote (Grupe & Nitschke, 2013; Mueller et al., 2010). For example, individuals with an extremely anxious or inhibited temperament may miss out on opportunities to build enriching peer relationships for fear that they will eventually be rejected. This isolation can, in turn, exacerbate anxiety and depression (Rubin et al., 1989; Vernberg et al., 1992). Understanding the neurobiology that underlies these temperaments and contributes to the selection of anxious emotional responses to innocuous stimuli could lead to new behavioral and pharmacological interventions that bring relief to millions.

Human Neuroimaging Studies Identify the Neural Circuitry of Inhibited Temperament

Since the functional neuroimaging revolution of the late 20th century (Roalf & Gur, 2017), clinical and basic research communities have made great strides toward identifying the neural circuits that contribute to extremely anxious or inhibited temperaments and the stress-related psychopathology they predict (Blackford & Pine, 2012; Chavanne & Robinson, 2021). By combining functional neuroimaging methods such as fMRI with anxiogenic paradigms like classical conditioning (e.g., Rehman et al., 2021), traumatic recall (e.g., Rahman & Brown, 2021), and symptoms provocation (e.g., Shin & Liberzon, 2010), researchers have identified a distributed network of regions that are differentially engaged during threat processing in pathologically anxious and nominally healthy individuals, including several cortical, subcortical, and midbrain structures. A meta-analysis of 181 fMRI studies revealed that an overlapping circuit—including the amygdala, bed nucleus of the stria terminalis (BST), periaqueductal gray (PAG), midcingulate cortex (MCC), and anterior insula (AI)—is preferentially recruited by people who suffer from anxiety disorders in response to emotional challenges and by healthy volunteers during threat conditioning and uncertain threat anticipation tasks (Chavanne & Robinson, 2021). This body of work indicates that heightened threat sensitivity across a coordinated circuit is a transdiagnostic feature of anxiety disorders. The assortment of regions comprising this circuit underscores the deep sensory, contextual, and regulatory integration necessary to adaptively evaluate and respond to threats.

Adaptive Threat Processing Requires Central Integration and Adjudication

The abundance of regions implicated in threat processing should come as no surprise. Every brain region has evolved to optimize survival, and owing to strong selection pressures like predation, most brain regions are involved in balancing risks against potential rewards (Blanchard et al., 2011; Blanchard & Blanchard, 2008; Mobbs et al., 2009, 2015). Our brains are constantly interpreting an immense volume of information pertaining to our homeostatic states, interoceptive signals, memories, surroundings, expectations, subjective value judgments, and so on, in what we might conceptualize as an *n*-dimensional threat-processing feature space (Holley & Fox, in press). In this feature space, tilting the weight of a specific feature might nudge the probability of action selection toward one behavior or another. The features—let alone the weights—inherent to the selection of any behavior are challenging to enumerate, yet our brains arbitrate between a dizzying number of these selections at any given time. Indeed, anytime we respond to survival-relevant stimuli, we engage in the process of *selecting* an emotional response to it. Despite the complexity of the threat-processing feature space, action plans are swiftly developed and implemented. They must be. With survival on the line, a good plan executed now is often better than the perfect plan executed a moment too late. Think back to our grazing gazelle. Perhaps it hears a noise in the tall grass but doesn't yet see a predator. Does fleeing immediately, and thereby foregoing the remainder of its meal, offer the best risk-versus-reward trade-off? The somewhat unsatisfying answer is, "It depends" (Cooper & Blumstein, 2015; Evans et al., 2019). How hungry is the gazelle? Has it previously encountered a predator in this area? Is the grazing patch high- or low-quality? Does the terrain favor a last-moment escape? Is food generally hard to come by, or abundant? These are only a sampling of the factors that shape the threat-processing feature space unique to that gazelle at that exact moment, and a multitude of brain regions are engaged in encoding their values. How might the brain mitigate "paralysis by analysis"

in such an information-rich, time-sensitive scenario, in which most if not all brain regions are vested parties? One elegant solution is through the centralized selection of emotion-relevant responses whose downstream cascades rapidly promote the physiological and behavioral repertoires necessary to overcome challenges and seize opportunities. To be sure, several brain regions feature evolved mechanisms for selecting emotion-relevant responses in certain contexts—a point that we revisit later. However, the EAc—a distributed neuroanatomical concept that includes the central nucleus of the amygdala (Ce) and the BST—stands out as uniquely well-suited to the general task of selecting between competing emotion-relevant responses in a variety of survival-relevant contexts.

The EAc receives robust polymodal inputs – both direct and indirect – from regulatory/evaluative regions including the prefrontal cortex and AI, contextual regions such as the hippocampus, and sensory regions like the thalamus (de Olmos & Heimer, 1999; Fox et al., 2015). It rapidly translates these inputs into emotion-relevant responses that promote fitness-optimizing behaviors under the dynamic constraints of the moment (Mobbs et al., 2015) and launches those behaviors into action via dense projections to downstream effector regions like the reticular formation and the PAG (*Figure 2*; Fox et al., 2015; Shackman & Fox, 2016). The EAc's major components, the BST and the Ce, are deeply implicated in orchestrating defensive responses to a variety of threats. These nuclei form a tightly interconnected, distributed circuit. They share similar patterns of connectivity (Oler et al., 2017; Rabellino et al., 2018; Roy et al., 2009), cellular composition (McDonald, 1982, 1983), neurochemistry (Gray & Magnuson, 1992), and gene expression (Bupesh et al., 2011; Lein et al., 2007). These qualities position the EAc at the center of what Mobbs and colleagues have aptly dubbed the *Survival Optimization System* (Mobbs et al., 2015)—a distributed network that has evolved to promote fitness in challenging contexts. As one might predict, dysregulation of the EAc leads to impaired selection of emotion-relevant responses and promotes maladaptive behavior. In patients with bilateral amygdala damage, these impairments are often profound.

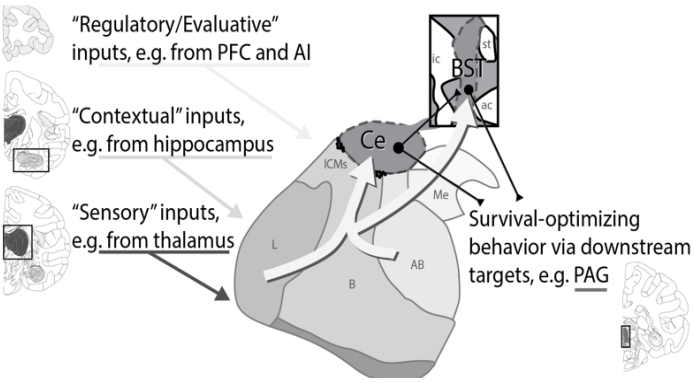

Figure 2: Simplified schematic showing converging inputs from regulatory/evaluative, contextual, and sensory regions onto the amygdaloid complex, where information is rapidly integrated by the EAc to select emotion-relevant responses, with associated behaviors carried out by downstream effector regions. (Ce: central nucleus of the amygdala; BST: bed nucleus of the stria terminalis; PFC: prefrontal cortex; AI: anterior insula; ICMs: intercalated masses; L: lateral amygdala; B: basal amygdala; AB: accessory-basal amygdala; Me: medial amygdala; st: stria terminalis; ac: anterior commissure; ic: internal capsule.) Adapted from Fox & Kalin, 2014.

A "Fearless" Patient Offers Insights into the Amygdala's Role in Human Behavior

In a famous series of studies beginning in the mid-1990s, researchers at the University of Iowa's Department of Neurology described the case of Patient S.M., who suffers from Urbach-Wiethe

disease—an *extremely* rare autosomal recessive disorder that causes bilateral calcification of the patients' amygdaloid complex, including the EAc's Ce component (Adolphs et al., 1995; Aggleton, 2000). S.M.'s condition afforded researchers the unique opportunity to study a patient who exhibited a complete loss of amygdala function, but whose brain was otherwise healthy and intact. Consistent with the idea that the amygdala is central to threat processing, Patient S.M. showed blunted responses to a range of threat stimuli. For example, although S.M. self-reported a fear of snakes, she readily handled a snake whenever asked (Feinstein et al., 2011). She showed little regard for personal space, and was described as an extremely "close talker," even when speaking with strangers (Kennedy et al., 2009). S.M. was unable to recognize fearful, threatening, or untrustworthy faces (Adolphs et al., 1994, 1998); to draw a facial expression of fear (despite being a competent artist who could sketch other facial expressions of emotion; Adolphs et al., 1994; Aggleton, 2000), or to identify the affective quality of "scary" music (Gosselin et al., 2007). Underscoring her impaired threat-processing faculties and incapacity for fear learning (Bechara et al., 1995), S.M. had repeatedly been victimized by violent criminals, and yet she showed no signs of post-traumatic stress or increased vigilance (Feinstein et al., 2011). In gambling tasks, S.M. was far more willing than controls to accept disadvantageous propositions, such as even odds of winning $20 versus losing $50 (De Martino et al., 2010). In sum, S.M. appeared largely unable to experience adaptive fear or anxiety (though see Khalsa et al., 2016, for evidence of induced panic in Urbach-Wiethe patients through cardiorespiratory interoceptive pathways).

What can we confidently infer about brain function from studies documenting Patient S.M.'s salient behavior anomalies? Unfortunately, less than we would like. Urbach-Wiethe disease is exceptionally rare, and only a few hundred cases have been documented in the extant medical literature (Kabre et al., 2015). Moreover, some studies have characterized the behavior of other Urbach-Wiethe patients quite differently from S.M.'s, though in these patients the Ce appears to have been spared (Terburg et al., 2012). Furthermore, S.M. was first evaluated *after* her amygdalae became calcified by the disease. Consequently, descriptions of her premorbid behavior are based on personal recall and report, not on empirical measurement. These factors make findings from studies of S.M. difficult to interpret. Although it may be tempting to do so, we cannot infer that S.M.'s maladaptive risk-taking behavior is *caused* by her amygdala damage. Induced lesion studies with pre- and post-morbid measures can support claims of causality, but naturally such research is nonviable in human volunteers. Fortunately, the circuits that underlie anxious and inhibited temperament and govern the selection of emotion-relevant responses in humans are conserved in our closest evolutionary relatives, nonhuman primates.

Nonhuman Primates Enable Translational Investigations of Threat-Processing Circuits

Nonhuman primates are our closest phylogenetic neighbors, and our shared homology, genetic makeup, and sociobehavioral repertoires are critical enablers of high-impact translational neuroscience. *Homo sapiens* and rhesus monkeys (*Macaca mulatta*) diverged from our common evolutionary ancestor only 25 million years ago (compared to 75 million years ago for mice/rats). Numerous features of our gross and brain anatomies are well conserved, including a highly elaborated prefrontal cortex (Barbas, 1995; Barbas et al., 2011; Barbas & Pandya, 1989; Ongür & Price, 2000). We share a roughly 93-percent genetic overlap with *M. mulatta* (Rhesus Macaque Genome Sequencing and Analysis Consortium, 2007). Importantly, we have similar

developmental trajectories, social structures, and behavioral tendencies (Kalin & Shelton, 2003; Phillips et al., 2014). Our biobehavioral responses to threat are also highly conserved (Kalin, 2002; Kalin & Shelton, 1989), which affords nonhuman primate researchers unique insights into disorder-relevant neurobiology.

Functional Neuroimaging in Rhesus Monkeys Reveals Conserved Threat-Processing Substrates

As is the case with human studies, functional neuroimaging studies of nonhuman primates have revealed a distributed threat-processing circuit centered on the EAc and encompassing the PAG, MCC, and AI, indicating that threat-sensitive substrates are highly conserved in humans and nonhuman primates (Fox et al., 2015; Kalin & Shelton, 2003; Oler et al., 2012, 2017). Our group investigates these substrates in a well-validated rhesus model of early life anxious temperament (AT) using multimodal functional neuroimaging and an ecologically relevant anxiogenic assay: the *No Eye Contact* context of the Human Intruder Paradigm, or *NEC* (Fox & Kalin, 2014; Gottlieb & Capitanio, 2013; Kalin & Shelton, 1989; Oler et al., n.d.). During the NEC, an unfamiliar human intruder enters the room and presents their profile to an individual, caged animal without making eye contact. When faced with a diffuse, uncertain threat (such as a large, unfamiliar creature that has yet to spot you) it is adaptive to freeze and stay quiet to avoid being noticed. We have measured and related these defensive behaviors to additional measures of personality (including AT) and brain function (including regional metabolism and functional connectivity) in hundreds of 2- to 3-year-old rhesus subjects. Our use of the NEC with subsequent 18-fluorodeoxyglucose (FDG)-facilitated positron emission tomography (PET) has revealed integrated metabolism throughout a distributed network of threat-sensitive brain regions—including the EAc, PAG, MCC, and AI—as correlate of the anxious phenotype. Compared to animals with lower EAc metabolism, animals with higher EAc metabolism tend to freeze more, vocalize less, and show a greater increase in blood cortisol levels when exposed to the diffuse, uncertain threat represented by the NEC context (Fox et al., 2012, 2015; Fox & Kalin, 2014; Oler et al., n.d.; Shackman et al., 2013). Moreover, our pedigree-based heritability analyses have shown that BST metabolism is co-inherited with AT (Fox et al., 2018). Thus, it appears that the risk to develop anxiety disorders, inferred from high AT, is conferred via genetic influence on BST metabolism.

In addition to measures of integrated metabolism, our research group has explored functional connectivity between the Ce and BST in rhesus subjects using a multimodal neuroimaging approach (i.e., fMRI and FDG-PET) coupled with pedigree analyses. In studies using a large (N=378) multigenerational cohort, we found Ce-BST functional connectivity during light anesthesia to be heritable and associated with significant variance in AT (Fox et al., 2018, 2021). Furthermore, we found that elevated Ce-BST functional connectivity (measured via fMRI) predicted higher integrated metabolism (measured via FDG-PET) in the hypothalamus and PAG—downstream targets of the EAc that initiate neuroendocrine and behavioral responses to threat, respectively. Notably, although pedigree analysis revealed that both BST metabolism and Ce-BST functional connectivity were significantly correlated with animals' genetic relatedness, BST metabolism and Ce-BST functional connectivity were not correlated with each other. These findings suggest that there may be multiple mechanisms by which cells in the EAc mediate the inherited risk for psychopathology.

Together, these findings suggest that a distributed circuit, centered on the EAc and conserved in humans and nonhuman primates, is preferentially involved in mounting adaptive responses to threat. Moreover, they open the door to targeted lesion studies in nonhuman primates to test the EAc's causal contributions to individual differences in risk-taking and reward-seeking behaviors.

EAc Lesions in Rhesus Induce an "S.M.-like" Phenotype

Lesions of the amygdaloid complex in rhesus monkeys produce a range of aberrant behaviors reminiscent of Patient S.M. Compared to unoperated controls, animals that undergo bilateral neurotoxic lesioning of the amygdaloid complex exhibit more affiliative and sexual behaviors toward unoperated control animals in dyadic interactions (Emery et al., 2001). Furthermore, amygdala-lesioned animals engage in more affiliative and social behaviors toward control animals in studies of four-animal interactions (Machado et al., 2008). Bilateral amygdala lesions also affect foraging behavior, such that lesioned rhesus subjects are far more likely to consume unpalatable foods than unoperated controls (Machado et al., 2010). Additionally, lesioned animals attend to and interact with potentially dangerous objects more readily than unoperated controls (Bliss-Moreau et al., 2010, 2011; but see Charbonneau et al., 2021). These findings support a role for the amygdala in the maintenance of rhesus' species-typical emotional responses, such as wariness of novel conspecifics and rejection of potentially dangerous food sources, and show that the loss of amygdala function induces an "S.M.-like" phenotype in one of our closest phylogenetic relatives. Since the amygdaloid complex consists of many nuclei, however, these animal studies beg the question of which nuclei may be responsible for certain behaviors.

Spatially precise studies in which the *central* amygdalae of nonhuman primates are ablated underscore the causal relationship between Ce activity and the ability to adaptively respond to threats. In an influential study, Wisconsin researchers compared the impact of excitotoxic Ce lesions on fear- and anxiety-like behavior in rhesus (Kalin et al., 2004). The researchers exposed 30 monkeys in total—9 with bilateral Ce lesions, 5 with unilateral Ce lesions, and 16 unoperated controls—to two anxiogenic paradigms: one involving a snake (often regarded as an innately threatening stimulus; Isbell, 2006; Weiss et al., 2015), and the other an unfamiliar human intruder. Consistent with our view that the Ce promotes adaptive anxiety that constrains gratuitous risk-taking, the bilateral lesion group showed significantly reduced threat sensitivity in these contexts compared to unilaterally lesioned and control animals. In the first context, animals had to reach past a live Northern pine snake (*Pithucus melanoleucusi*) to obtain a food reward—a task that highly inhibited rhesus subjects are less likely to perform (Fox et al., 2021). Bilaterally lesioned animals were quicker to reach past the snake, suggesting impaired emotion-response selection (i.e., an inability to exhibit anxiety, when anxiety is adaptive). In the second context, each animal was placed in a small enclosure and exposed to the NEC. As in the snake task, bilaterally-lesioned animals froze far less often in the presence of the human intruder than unilaterally lesioned monkeys and controls, implicating the Ce in the ability to mount adaptive threat responses.

In addition to evaluating the impacts of Ce lesions on risk-vs-reward trade-offs and defensive responding in rhesus monkeys, the Wisconsin primate research group also investigated the relationship between threat sensitivity and neuroendocrine activity by measuring concentrations

of corticotropin releasing hormone (CRH; often referred to as corticotropin releasing *factor*, or CRF) in the cerebrospinal fluid (CSF) of lesioned and intact animals immediately following exposure to the NEC context. Having previously observed that CRH concentrations in the CRF were proportional to the number of anxiety-like behaviors exhibited by intact animals, Kalin et al. (2004) examined whether Ce damage *causes* reduced CSF CRH titers. Consistent with prior correlational findings, rhesus subjects with bilateral Ce damage had significantly lower CSF CRH concentrations than unilaterally lesioned animals and unoperated controls. These findings established the EAc's causal role in engaging the hypothalamic-pituitary-adrenal axis in the promotion of adaptive stress physiology. Taken together, these physiological and behavioral findings are suggestive of a blunted ability to select an adaptive, anxious emotional response to threat.

Lesion studies are especially valuable for establishing causation and helping to bridge the gap between neuroimaging and molecular research (but see Bliss-Moreau et al., 2017, for an insightful discussion on challenges to the inferences that can be made in lesion studies). In the following section, we discuss how genetic and molecular techniques complement lesion studies and are used to great effect in nonhuman primate investigations of anxious and inhibited temperament.

Genetic and Molecular Findings in Rhesus Hint at Anxiety's Complex Neurobiology

The nonhuman primate neuroimaging and lesion studies discussed thus far have (1) revealed the presence of a distributed threat-processing network, centered on the EAc, in both humans and nonhuman primates; (2) linked heightened EAc metabolism and functional connectivity to extremely anxious or inhibited temperaments; and (3) established the Ce's causal role in the promotion of adaptive threat response. However, cell populations in the Ce and BST are deeply heterogeneous (Gungor & Paré, 2016; Janak & Tye, 2015; Lebow & Chen, 2016), and identifying the genetic and molecular markers characteristic of this at-risk phenotype is beyond the capability of functional neuroimaging (Fox & Shackman, 2019). Considering that the smallest unit of spatial resolution captured by fMRI or PET scans may represent the activity of several hundred thousand neurons, it is impossible to infer which neurons—and by extension which molecules and neurotransmitters—might be principally responsible for the relationship between a voxel's activation and associated defensive behaviors. Yet such insights are critical to the development of clinical entry points that lead to novel treatment approaches. Fortunately, nonhuman primate models of anxious and inhibited temperament are increasingly amenable to genetic and molecular inquiry.

Motivated by the neuroimaging and lesion findings described above, researchers have used genetic techniques to shed new light on the neurobiology of anxious behaviors in studies that are paving the way toward new treatment approaches. Indeed, viral-mediated gene overexpression studies with rhesus monkeys appear especially promising as a translational approach to the development of personalized medicine (Bulcha et al., 2021). In one such study, researchers used a vector-mediated gene delivery approach by introducing an engineered adeno-associated virus (AAV) to express CRH above endogenous levels in the bilateral dorsal amygdalae (which encompasses the Ce) of five young, male rhesus monkeys (Kalin et al., 2016). In line with the previous findings, animals with dorsal amygdala CRH overexpression scored higher in

subsequent composite measures of anxious temperament during the NEC. Additionally, PET scans revealed that dorsal amygdala CRH overexpression resulted in greater pre- versus post-experiment metabolic increases in the dorsal amygdala region, as well as regions of the hippocampus, AI, and orbital proisocortex. These results demonstrate that manipulating the expression of a specific molecule (in this case, CRH) in the dorsal amygdala can alter threat processing and influence the function of a distributed neural circuit known to be involved in human anxiety.

CRH is likely one of many molecular footholds on the climb toward clinical breakthroughs. Several other candidate molecules and neurotransmitters show great clinical promise, and nonhuman animal studies will be instrumental to their preclinical development. Moreover, discovery-based approaches in nonhuman animals will continue to unearth new candidate molecules. For example, in a discovery-based study of 46 young rhesus monkeys (Fox et al., 2019), our research group used RNA sequencing to search for associations between alterations in gene expression in the dorsal amygdala and anxious temperament. *NTRK3*, a neurotrophic factor related to intracellular neuroplasticity pathways, showed an inverse association with anxious temperament, such that more anxious animals exhibited lower *NTRK3* expression. To evaluate the causal relationship between *NTRK3* function and anxious temperament, we administered an AAV to produce overexpression of *NTRK3*'s endogenous ligand, neurotrophin-3 (NT-3), in the dorsal amygdalae of five animals. Pre- and post-operative NEC measures of anxious temperament and associated brain metabolism (via FDG-PET) were obtained. Viral overexpression of NT-3 significantly reduced composite measures of anxious temperament, notably by reducing the time spent freezing in response to the human intruder, and significantly altered brain metabolism across a distributed network of regions that included the Ce and BST. Intriguingly, these NT-3/NTRK3 findings hint toward a general anxiolytic effect of inducing plasticity in threat-sensitive brain circuits. (This inference has been strengthened by recent mouse research documenting increased plasticity in CRH Ce neurons following threat conditioning; see Botta et al., 2015.) In another discovery-based study, Kovner and colleagues (2020) used RNA sequencing of tissue obtained from lateral Ce (CeL) neurons of 47 young rhesus monkeys to identify 14 transcripts that were significantly associated with anxious temperament. Protein kinase C-delta (PKCδ)— a marker for cell-types implicated in anxiety-like behavior in mice (Botta et al., 2015; Haubensak et al., 2010)—was among the significant transcripts. Building on our previous observation that inherited Ce-BST functional connectivity was associated with anxious temperament (Fox et al., 2018), Kovner et al. examined the relationship between PKCδ+ cells and Ce-BST projections. These investigators found that PKCδ+ cells represented the majority of BST-projecting CeL neurons, suggesting a mediating role for PKCδ in heritable alterations in Ce-BST functional connectivity. As noted in the concluding section of this chapter, converging evidence from nonhuman primate and rodent models suggests that Ce PKCδ+ neurons may be a clinical entry point in the development of new interventions for anxiety disorders.

The foregoing findings underscore the importance of nonhuman primate models in both theory-driven and discovery-based studies of the neurobiology that promotes anxious and inhibited temperaments characteristic of anxiety disorders. Our close evolutionary kinship with nonhuman primates increases confidence that this neurobiology, and the systems and behaviors it engages,

are highly conserved. Fully understanding the mechanisms of these substrates, however, requires model organisms that support spatio-temporally precise and cell-type-specific manipulations of the EAc's *microcircuitry*—that is, the organization of neurons into local information-processing units (Shepherd, 2011). Rodent models of anxiety are particularly appropriate to support these investigations.

The Value of Rodent Models in the Study of Anxiety Disorders

Rodents are the most widely used laboratory animals (Hickman et al., 2017). Compared to nonhuman primates, mice and rats are relatively easy and inexpensive to house, care for, and breed (Bryda, 2013; Hickman et al., 2017). We diverged from a common ancestor roughly 75 million years ago, and the protein-coding regions of our DNA are about 85-percent identical to those of mice (Mouse Genome Sequencing Consortium, 2002). Thus, they are an outstanding model for high-throughput generation and refinement of specific translational hypotheses, as well as the development of clinical targets. Furthermore, many of the brain regions and molecular substrates implicated in anxiety disorders are conserved in rodents (Chareyron et al., 2011; Mantini et al., 2013; Ongür & Price, 2000; Phillips et al., 2014), as are the generalities of our threat-processing repertoires (e.g., freezing in response to uncertain potential threats; Blanchard et al., 2011; Kalin, 2002; Roelofs, 2017). Accordingly, rodent research has been instrumental in unraveling the mysteries of the EAc's threat-sensitive microcircuitry and its role in mediating the selection of adaptive emotion-relevant responses.

Insights from Rodent Studies into the Ce Microcircuits that Drive Defensive Responding

The Ce, a core component of the EAc, is itself divisible into lateral (CeL), medial (CeM), and capsular subregions (Cassell et al., 1999). These subnuclei form local microcircuits, often consisting of intermingled cell populations. Rodent studies are well-suited for investigating the role these microcircuits play in defensive responding, since such investigations benefit greatly from the targeted manipulation of specific cell populations. For example, in the CeL, genetically distinct populations of PKCδ-negative (PKCδ-) "CeL_{on}" and PKCδ+ "CeL_{off}" neurons form a reciprocal inhibitory microcircuit that gates conditioned freezing via projections to the CeM (Botta et al., 2015; Ciocchi et al., 2010; Haubensak et al., 2010). The tonic firing rate of PKCδ+/CeL_{off} neurons increases following fear conditioning, and this increase corresponds to an increase in threat generalization characteristic of anxiety disorders. Furthermore, optogenetic activation of these neurons increases anxiety-like behaviors in anxiogenic assays like the elevated plus maze, whereas optogenetic inhibition produces the opposite effect. These results complement Kovner et al.'s (2020) study of the nonhuman primate CeL PKCδ's role in anxiogenesis and demonstrate how converging mouse and nonhuman primate findings strengthen our understanding of the neural substrates that underlie anxious pathology.

Rodent models can also answer key questions about the mechanisms involved in rapid switching between defensive responses, potentially offering further insight into why anxious individuals sometimes feel and behave as though they are paralyzed by threats (Schmidt et al., 2008). Since optogenetics (Deisseroth, 2011) affords researchers millisecond-scale temporal resolution and bidirectional control over genetically defined cell populations, it provides an effective method for investigating fast action selection between mutually exclusive behaviors—for example, the neural

processes that an animal to freeze or flee. In light of considerable evidence that the EAc serves as the brain's arbitrator of survival-optimizing trade-offs, it stands to reason that some of its microcircuits should facilitate rapid, “winner take all” action selection. Optogenetics studies indicate that this is indeed the case. In mice, for example, CRH+ Ce neurons form one such microcircuit with an intermingled population of somatostatin-expressing (SST+) Ce neurons (Fadok et al., 2017). Researchers have used *in vivo* optogenetics to selectively target and activate these Ce neuron populations during threat assays and found that CRH+ neurons promote conditioned flight whereas SST+ neurons promote conditioned freezing. The activation of either neuronal population produces strong inhibitory postsynaptic currents in the *other* population. This latter finding is particularly relevant to the question of how the Ce might rapidly switch between mutually exclusive defensive behaviors like fleeing and freezing (*Figure 3*). Fadok et al. showed that either behavior is initiated via a “winner take all” strategy that relies on the “recurrent and reciprocal inhibitory interactions" (p.142) of closely intertwined neuronal subpopulations that promote one behavior and actively suppress the other.

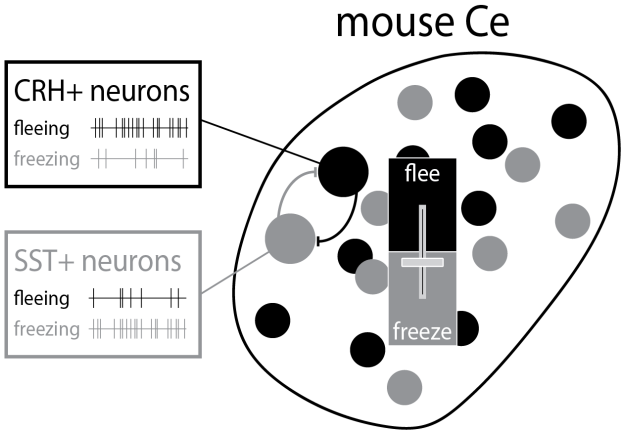

Figure 3: Simplified schematic of the mouse Ce microcircuit in which CRH+ and SST+ neuron activity promotes rapid switching between conditioned fleeing and freezing, respectively, via reciprocal inhibition. When Ce CRH+ neurons fire, they inhibit Ce SST+ neurons, and vice versa. Behavior promoted by the “winning” population is initiated through Ce projections to the PAG. Other “winner take all” mechanisms like this may be widespread throughout the EAc’s microcircuitry.

It bears mentioning that defensive microcircuits featuring other mechanisms, apart from recurrent and reciprocal inhibition, may have evolved in numerous regions within the brain’s distributed threat-processing network. For example, a calcium imaging and optogenetics study evaluating the relationship between threat saliency, escape behavior, and synaptic escape-threshold computations in mice found that a feed-forward recurrent *excitatory* microcircuit originating in the dorsomedial superior colliculus (dmSC) and projecting to the dorsal region of the PAG (dPAG) is involved in initiating escape (Evans and Stempel, et al., 2018). In this study, the salience of a simulated looming predator (i.e., an aversive overhead disc of various contrasts between 27 and 98 percent) drove concomitant increases in the strength of dPAG-projecting dmSC neurons’ sustained ensemble activity, such that increasing “predator” salience caused proportionate increases in firing activity throughout this microcircuit. Once the strength of the microcircuit’s ensemble activity reached an escape-initiation threshold, the animals’ responses to the threat immediately switched from freezing to fleeing.

In sum, rodent models and optogenetics studies have proven valuable for investigating the neural microcircuitry that underlies the defensive aspects of anxiety-like behavior, such as freezing and escape. Beyond revealing the mechanisms of rodent neurobehavioral threat sensitivity, the

studies we have described above form an excellent foundation for work in nonhuman primates by offering hints about key facets of anxiety-related neurobiology may be conserved across species. Collectively, these studies will be instrumental in resolving what we believe to be an understudied, but vitally important, function of ostensibly defensive circuitry: its role in reward-related processes.

Rodent Research Brings the EAc's Reward-Related Processes into Clearer Focus

Although the EAc is involved in a range of appetitive and consummatory behaviors, these functions have, until recently, been obscured by decades of intensive focus on the amygdala as the brain's putative "fear generator" (and, more recently, years of focus on the BST as its "anxiety generator"). The expansive literature treating the amygdala and BST as functionally distinct has been influential: For example, the National Institute of Mental Health's *Research Domain Criteria* (RDoC) framework, which encourages new approaches to the investigation of mental-health disorders, associates the Ce with "acute threat (fear)" and the BST with "phasic threat (anxiety)." However, numerous recent studies have called this functionally segregated view into question (e.g., Coaster et al., 2011; Fox & Shackman, 2019; Mobbs et al., 2007, 2010; Shackman & Fox, 2016; Somerville et al., 2010), and mounting evidence supports our position that the Ce and BST function in concert, as a distributed circuit capable not only of assembling fearful and anxious states but also of selecting between competing emotional responses to survival-relevant challenges and opportunities more broadly.

Studies of predatory hunting behavior in rats, for instance, have uncovered a bifurcated appetitive-consummatory circuit projecting from the Ce to the PAG and parvocellular reticular formation (PCRt; Han et al., 2017). Optogenetic stimulation of Ce-originating synaptic terminals in the PAG elicits immediate hunting behavior in rats in the presence of live or artificial prey, whereas activation of Ce-originating terminals in the PCRt induces biting attacks against those same targets, as well as "fictive feeding" behavior in the absence of food, prey, or inedible objects. Perhaps most fascinatingly, activation of the PCRt pathway *does not* promote biting attacks against conspecifics, nor does it increase anxiety-like behaviors, highlighting the context dependency of these circuits, and a broader role for the EAc in behaviors that are not associated with fear and anxiety. Another example of context dependency, related to feeding, is that chemogenetic inhibition of CeL PKCδ+ neurons—which, as we discussed in the preceding section, are involved in threat conditioning—leads to risky feeding behavior, such as the consumption of bitter (and thereby potentially dangerous) foods (Cai et al., 2014; Ponserre et al., 2020). Studies of appetitive motivation also hint at the EAc's sensitivity to context. For instance, Kim et al. (2017) reported that mice chose to self-stimulate for optogenetic activation of Ce CRH+ neurons, despite the role these neurons play in promoting conditioned flight following fear learning. Another study by Baumgartner et al. (2021) found that optogenetic activation of Ce CRH+ neurons increased rats' incentive motivation (measured as their willingness to work for sucrose rewards in operant tasks) whereas optogenetic activation of BST CRH+ neurons decreased motivation. Together, these findings highlight the EAc's role in reward-seeking behaviors, complementing decades of fear- and anxiety-related research into these regions and reinforcing our view that this circuit plays a foundational role in the selection of adaptive emotional responses to a range of survival-relevant contexts, and not merely to threats.

At this juncture, it is important to remember that the EAc lies at the center of a distributed network of brain regions that collectively encodes information and dynamically shapes the threat-processing feature space we envisioned above (Chavanne & Robinson, 2021; Mobbs et al., 2009, 2015; Roelofs, 2017; Tovote et al., 2015). Although we view the EAc as the centralized integrator of this information, alterations throughout the distributed network are liable to produce aberrant processing. The basolateral amygdala (BLA), for example, is adjacent to the Ce and sends dense projections to the Ce and BST (Dong et al., 2001; Swanson & Petrovich, 1998). Researchers in the post-traumatic stress disorder (PTSD) area have long been interested in the BLA due to its documented role in fear-learning and -extinction processes (Gale et al., 2004; Sharp, 2017; Terburg et al., 2012). In recent years, memory researchers have uncovered a mouse microcircuit consisting of two groups of spatially segregated BLA neurons that exhibit feed-forward reciprocal inhibition via local interneurons in the assembly of opposing affective and behavioral states: R-spondin 2-positive (Rspo2+) neurons, which respond to negatively valenced stimuli and mediate negative behaviors and memories, and protein phosphatase 1-regulatory inhibitor subunit 1B-positive (Ppp1r1b+) neurons, which respond to positively valenced stimuli and mediate appetitive behaviors and memories (Zhang et al., 2020). Optogenetic and histological studies of this microcircuit during fear-extinction paradigms have revealed that fear extinction promotes the formation of new Ppp1r1b+ neurons. Intriguingly, these newly formed neurons are functionally indistinguishable from the *reward*-responsive BLA neurons of control animals that have not been exposed to fear-learning or -extinction protocols. These findings hint toward the possibility that the omission of an expected threat stimulus is intrinsically rewarding. From a clinical perspective, PTSD patients' theorized inability to extinguish fear memories may relate to alterations in this microcircuit that prevent the formation of new Ppp1r1b+ neurons, wherein (rewarding) fear-extinction memories are stored. The use of rodent models to resolve the fine mechanistic aspects of not only the EAc's major components, but also the brain's distributed threat-processing network more broadly, will grow increasingly impactful as precision treatment approaches capable of targeting specific mechanisms and pathways mature (Li & Auwerx, 2020; National Academies of Sciences, 2018).

Conclusions

In this chapter, we have argued that anxiety disorders reflect an impaired ability to select adaptive emotion-relevant responses to the challenges and opportunities in our environments, and that this impairment underlies extremely anxious or inhibited temperaments—known risk factors for the development of anxious pathology. The circuits that govern the selection of these emotion-relevant responses are present in numerous mammalian species, including nonhuman primates and rodents, enabling investigations that collectively span multiple, overlapping levels of analysis—from complex social interactions among groups, to the brain circuits, cell types, genes, and molecules that influence those interactions. Such investigations are reshaping our understanding of anxiety disorders and unraveling the mysteries of their transdiagnostic features.

In practice, these investigations are forming a robust framework that guides our approach to basic and clinical research by enabling the generation of specific, clinically relevant hypotheses. For example, our RNA-sequencing studies suggest that individual differences in anxious and inhibited temperaments are partially mediated by alterations in CeL PKCδ+ neurons (Kovner et al., 2020).

A subset of these neurons projects to the BST, suggesting that targeting PKCδ+ neurons may be a clinical entry point for treating individuals with heightened Ce-BST connectivity. Leveraging this knowledge in the treatment of anxiety will require developing new treatment approaches and determining if the source of these differences in anxious and inhibited temperaments results from increased *expression* of PKCδ in this subset of CeL neurons, or an increased *proportion* of CeL PKCδ+ neurons. Findings from studies with rhesus monkeys are consistent with the established role of CeL PKCδ+ neurons in rodent threat learning (Haubensak et al., 2010), and they provide an opportunity to translate these findings into humans.

Importantly, neuroimaging (Chavanne & Robinson, 2021; Fox et al., 2018) and genetic (Calboli et al., 2010; Genetics of Personality Consortium, 2015; Levey et al., 2020) studies underscore the heterogeneity of mechanisms that contribute to individual differences in the risk to develop anxiety disorders. Future studies should investigate complementary hypotheses stemming from our working theory that patterns of maladaptive emotion-response selection common to extremely anxious or inhibited individuals are caused by imbalances within EAc (or EAc-adjacent) circuitry. For example, an anxious individual might have a microcircuit imbalance in their Ce, such that SST+ neurons are overrepresented compared to CRH+ neurons. Such an imbalance could result in a persistent inhibition of CRH+ neurons by SST+ neurons, thereby biasing the individual toward maladaptive “freezing up” in response to mild challenges. On the other hand, circuit-level alterations in threat-sensitive substrates could be the culprit. For example, this anxious individual’s long-range connectivity patterns of EAc inputs could promote a bias toward “freezing up” by chronically misweighting socio-environmental threat cues like facial expressions of emotion. Alternatively, their EAc might have deficient inputs from threat-moderating neurons in regions like the prefrontal cortex, or excessive inputs from threat-sensitive neurons in regions like the paraventricular thalamus or lateral parabrachial nucleus (Palmiter, 2018). Or perhaps alterations to this individual’s *reward*-sensitive EAc substrates are a factor. Indeed, research identifying context-sensitive EAc mechanisms that are differentially engaged in *both* threat- *and* reward-processing suggests that the neurobiological substrates of stress-related psychopathology are more nuanced than previously imagined (Cai et al., 2014; Han et al., 2017; Kim et al., 2017; Ponserre et al., 2020; Zhang et al., 2020).

To bring much-needed relief to millions who suffer from anxiety disorders, hypotheses like these must be refined and tested. Nonhuman animal research has been, and will remain, indispensable to this effort. By delivering an improved understanding of the mechanisms that underlie extremely anxious or inhibited temperaments, nonhuman animal research will continue to reveal clinical targets and inspire novel approaches to treat and prevent psychopathology.

Acknowledgments

This work was supported by R01MH121735 to ASF, and the California National Primate Research Center (P51OD011107). DH thanks Kara Marszalek for her contributions and support.

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