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REPEATED THREAT (WITHOUT DIRECT HARM) ALTERS METABOLIC CAPACITY IN SELECT REGIONS THAT DRIVE DEFENSIVE BEHAVIOR

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Abstract—To understand the behavioral consequences of intermittent anticipatory stress resulting from threats without accompanying physiological challenges, we developed a semi-naturalistic rodent housing and foraging environment that can include threats that are unpredictable in timing. Behavior is automatically recorded while rats forage for food or water. Over three weeks, the threats have been shown to elicit risk assessment behaviors, increase defensive burying and increase adrenal gland weight. To identify brain regions activated by this manipulation, we measured cytochrome c oxidase (COX), which is tightly coupled to neural activity. Adolescent male Sprague–Dawley rats were randomly assigned to control (CT) or unpredictable threat/stress (ST) housing conditions consisting of two tub cages, one with food and another with water, separated by a tunnel. Over three weeks (P31–P52), the ST group received randomly timed (probability of 0.25), simultaneous presentations of ferret odor, an abrupt light, and sound at the center of the tunnel. The ST group had consistently fewer tunnel crossings than the CT group, but similar body weights. Group differences in COX activity were detected in regions implicated in the control of defensive burying. There was an increase in COX activity in the hypothalamic premammillary dorsal nucleus (PMD) and lateral septum (LS), whereas a decrease was observed in the periaqueductal gray (PAG) and CA3 region of the hippocampus. There were no significant differences in the anterior cingulate cortex, prefrontal cortex, striatum or motor cortex. The sites with changes in metabolic capacity are candidates for the sites of plasticity that may underlie the behavioral adaptations to intermittent threats.

Key words: stress, predator threat, cytochrome oxidase, metabolic capacity, hippocampus, periaqueductal gray.

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Abbreviations: 2-DG, 2-deoxyglucose; AHN, anterior hypothalamic nuclei; COX, cytochrome c oxidase; dACC, dorsal anterior cingulate cortex; HPA, hypothalamic–pituitary–adrenal; LS, lateral septum; MOD, mean optical density; mPFC, medial prefrontal cortex; PAG, periaqueductal gray; PMD, premammillary dorsal nucleus; ROIs, regions or interest.

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INTRODUCTION

There is a rich history of studying fear and anxiety from an ethological perspective. From this framework, clinical anxiety has been proposed as the inappropriate timing and exaggerated expression of otherwise adaptive responses to ambiguous or threatening stimuli (Rodgers, 1997; Rosen and Schulkin, 1998). Anxiety also plays an adaptive role. From this perspective, fear and anxiety are elicited in different environmental contexts (e.g., presence vs. potential presence of a predator), can be distinguished by the behavioral responses (escape vs. approach), and those respective responses are differentially sensitive to anxiolytics (Blanchard et al., 1997; Gray and McNaughton, 2000; McNaughton and Corr, 2004; Canteras et al., 2012). Integrating these proposals generates the hypothesis that fear is associated with a specific threat that is proximal in space and time, and produces a subset of defensive responses dominated by escape. Anxiety, in contrast, occurs when the potential for harm is elevated but distant in space or time, thus creating conditions of uncertainty that elicit risk assessment/approach behaviors (Blanchard et al., 1997; Misslin, 2003; McNaughton and Corr, 2004), which have been well-described in the rat (Blanchard et al., 1986). Many of these behaviors are indeed sensitive to anxiolytics. Specifically, risk assessment of novel or threatening stimuli, open-arm entries in the elevated plus maze and defensive burying, are sensitive to anxiolytics (Blanchard et al., 1993, 1997; Jimenez-Velazquez et al., 2006). Whereas the former are easily viewed as approach behaviors, defensive burying is not. However, it has been shown to share features with approach behaviors (Pinel et al., 1994). The relationship between the distance to harm and behavioral responses, described by some as the defense cascade, has inspired very recent proposals for an ethological basis of human fear and anxiety (Grupe and Nitschke, 2013; Kozlowska et al., 2015; Mobbs et al., 2015). Therefore, capitalizing on animal models that induce approach behaviors could further our understanding of anxiety.

Fear and anxiety are often considered forms of affect. It is instructive to remember that the James-Lange's theory of emotion proposes that external conditions elicit emotional motor programs. These are executed through the emotional motor system (Holstege, 1992) in contrast to the voluntary motor system. The emotional motor system coordinates autonomic and behavioral responses. Feedback from the execution of these motor programs drives the conscious awareness of these states, which

in turn, are weighed in decision-making (Damasio, 1996). Emotional motor programs are, therefore, the foundation upon which affect lies. To understand distinct emotions, and the conditions that initiate and sustain them, we need to understand the relationships between specific environmental factors, the motor programs they elicit, and the behavioral adaptations that are produced and maintained by these experiences.

To create environmental conditions that elicit emotional motor programs, we have developed an ethologically relevant habitat for rodents that allows us to control environmental parameters and measure the immediate reactions in addition to the longer lasting behavioral adaptations (Kim and Anderson, 2015). Our first step has been to use the habitat to present repeated unpredictable threats comprising simultaneous presentations of an abrupt sound, a flash of light and a puff of ferret dander odor. The latter is an innately aversive unconditioned stimulus to rodents (Masini et al., 2006) and has been shown to increase the activation of c-Fos in regions of the emotional motor system (Masini et al., 2005; Butler et al., 2011). The threat stimuli are presented in the central location of a tunnel that the rat must cross to obtain food and water, which are placed on opposite ends of the tunnel. The stimuli are presented when the animal is detected at the center, but occur unpredictably with a probability of 0.25. Previous work from our lab has shown that these stimuli and eventually the location where they are presented elicit risk assessment behaviors (Kim and Anderson, 2015), which fall into the category of approach behaviors that are associated with anxiety (McNaughton and Corr, 2004). After receiving threats in the central location of the tunnel, rats displayed significantly greater levels of stretch-attending, head scanning, and passive avoidance. In the habitat, the combined threat and disparate location of resources create an approach-avoidance conflict that is inherent to most foraging environments, including environments that humans navigate daily.

After removal from the stress condition, rats in the stress group spent more time defensively burying (Kim and Anderson, 2015). In rats, risk-assessment behaviors, like those elicited in the tunnels, and defensive burying, which shares features of risk assessment (Pinel et al., 1994), are approach behaviors likely serving to monitor threats in the ambiguous conditions and to calculate the optimal response strategy from moment to moment. The behaviors that changed could be categorized as emotional motor programs that comprise the risk assessment/approach features of anxiety. In the stress group, the acoustic startle response sensitized over testing sessions (manuscript in preparation), and memory differed from controls (Kim et al., 2017). These lasting behavioral adaptations are consistent with threat-related hypervigilance and impaired safety learning, which have also been proposed to be features of anxiety (Grupe and Nitschke, 2013).

Having produced a habitat with uncertainty and ambiguity about the elevated risk of predation that, in turn, effectively increased behaviors associated with anxiety, it will be valuable to identify regions differentially activated by our repeated threat condition. Such regions would be candidates for sites of the plasticity that underlie the disposition toward motor programs associated with anxiety. To do this, we used a metabolic mapping method that would allow the survey of many brain regions simultaneously. Although many investigators use c-Fos and 2-deoxyglucose (2-DG) to survey acutely activated regions, we sought a method that reflects neural activity specifically related to exposure to our continual living environment over an extended time period. Therefore, we chose to measure the activity of cytochrome oxidase (COX), which is the third step in the electron transport chain, and is tightly coupled to the production of adenosine triphosphate (ATP) (Skou, 1965; Wong-Riley, 1989; Hevner et al., 1992). COX has been utilized to map sites of plasticity related to behavioral tasks since it was demonstrated that COX activity was reduced in central sensory structures deprived of sensory input (Wong-Riley, 1989). This enzyme is up or down-regulated by a number of behavioral conditions, including voluntary wheel running (McCloskey et al., 2001), and various learning tasks (Poremba et al., 1997, 1998; Hu et al., 2005, 2006), including spatial learning (Conejo et al., 2010). Changes in COX histochemical reactivity represent changes in CO protein levels, which are regulated by subunit mRNAs and mtDNA (Hevner and Wong-Riley, 1990). This regulatory process takes longer than acute energy demands associated with task performance typically measured by 2-DG uptake. Accordingly, changes in COX histochemistry in the present study should represent the basal metabolic demands of the living environment.

In the present study, rats were housed in either the stress condition, described above, or in the control condition, which was designed to be identical, but without exposure to threat (Kim and Anderson, 2015). After three weeks in their respective conditions, all rats were euthanized and brains were fresh frozen, sectioned and reacted for COX. Regions of interest included regions known to be activated by exposure to a predator odor, including the medial nucleus of the amygdala (MeA) (Canteras et al., 1997), and regions of the hypothalamic defensive system (Masini et al., 2005; Cezario et al., 2008), also known as the medial hypothalamus zone. The latter is critical for the expression of innate defensive behavior directed toward predators, predator odor threat and contextual cues (Canteras et al., 1997; Canteras, 2002). Within that latter zone are three key regions, the anterior hypothalamic nuclei (AHN), ventromedial hypothalamic nucleus dorsomedial part (VMHdm), and dorsal premammillary nucleus (PMD). This zone is modulated by the prefrontal cortex, amygdala, and lateral septum (LS). It projects to the periaqueductal gray (PAG), which contains cells that are activated by predator odor (Dielenberg et al., 2001; Vianna et al., 2003), implicated in defense behaviors (Schenberg et al., 1990; Keay and Bandler, 2001; Litvin et al., 2007; Carvalho-Netto et al., 2009; Assareh et al., 2016), and activate autonomic and skeletal motor neurons. Thus, we hypothesized that these regions, which fall within the emotional motor system would be activated by our threat condition and undergo