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Active coping toward predatory stress is associated with lower corticosterone and progesterone plasma levels and decreased methylation in the medial amygdala vasopressin system

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ABSTRACT

An active coping style displayed under stress – which involves proactive investigatory responses toward environmental threats – has been associated with reduced vulnerability to psychiatric illness. However, the neurobiological determinants of coping styles are not well understood. When rats are exposed to a naturalistic stressor (cat fur) in a group, some individuals in the group show robust active investigation of the stimulus while others show a passive response involving retreat, immobility and close aggregation with conspecifics. Here we explored endocrine and epigenetic correlates of these contrasting coping styles. Male Wistar rats ($n = 48$) were exposed to cat fur in groups of 4 and the passive and active responders were identified and assessed for endocrine and epigenetic differences. Three days after the final cat fur exposure, active responders had substantially lower plasma levels of corticosterone and progesterone than passive responders. Plasma and testicular testosterone levels did not differ between active and passive responders. Active responders had markedly less methylation of the AVP CGCG promoter region located at base 4970 in the posterodorsal region of the medial amygdala but did not differ in the methylation status of the CCGG sequence located at base 2243. This is in agreement with prior research suggesting that AVP and progesterone act in opposition within the medial amygdala to modulate stress-related behaviors. The present study reports striking endocrine and epigenetic differences between active and passive responders, providing insight into potential systems involved in the manifestation of differing coping styles.

Introduction

Individual animals exposed to the same stressful situation often exhibit diversity in their behavioral responses to that situation. A common difference relates to active and passive coping styles. Active response styles involve proactive investigatory responses toward environmental threats, a more aggressive phenotype toward conspecifics, and less pronounced neural and physiological stress responses (Koolhaas et al., 1999, 2010). Conversely, passive response styles involve avoidance of environmental threats, reduced aggression toward conspecifics, and a more pronounced neural and physiological stress response (Koolhaas et al., 1999, 2010). In humans, a more active coping style is associated with reduced vulnerability to anxiety disorders and depression as well as better physical health, particularly cardiovascular health outcomes, whereas a more passive coping style is associated with heightened risk of developing mood and anxiety disorders as well as cardiovascular problems (Chiavarino et al., 2012; LeDoux and Gorman, 2001; Russo et al., 2012).

In recent work we studied variability in the responses of rats exposed to a naturalistic stressor, cat fur, in a large arena (Bowen and McGregor, 2014; Bowen et al., 2012, 2013; Kendig et al., 2011). When rats were exposed to cat fur in groups of 4 cagemates we observed a clear distinction between active and passive responders (Bowen et al., 2013). Active responders exhibit more frequent approaches toward the predator stimulus and show less conditioned fear to a context associated with the predator odor. Conversely, passive responders avoid the stimulus and spend prolonged periods huddling together in one of the corners of the arena. These traits are highly consistent across repeated exposures to cat fur and generalize to other situations.

Our studies indicate the amygdala–lateral septum system may play an important role in the defensive response to cat odor (Bowen et al., 2013). The posteroventral medial amygdala (MePV), in particular, is strongly activated by cat odor and has been identified as a critical pathway in the defensive response to predator odors (e.g. risk assessment and inhibition of locomotor activity and grooming) (Dielenberg and McGregor, 2001; McGregor et al., 2004). Crosstalk between the MePV and the neighboring posterdorsal medial amygdala (MePD) appears to play an important role in some of the acute and more enduring behavioral

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responses to predator odor exposure, such as inhibition of mating (Apfelbach et al., 2005; Takahashi, 2014). The MePD has recently been linked to coping style, with early life stress associated with reduced activity in the MePD and an enduring passive coping style (Nishi et al., 2013). Pathways involving the neuropeptide AVP that are centered around the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and lateral septum have received particular attention in studies on aggression and coping styles (Koolhaas et al., 1998, 2010; Veenema and Neumann, 2007).

There is a close relationship between aggression and coping strategy in both humans and animals, with a strong positive association existing between an active coping strategy and propensity to display offensive aggression (Koolhaas et al., 2010; Veenema and Neumann, 2007). As such, offensive aggression is often used as a measure of coping style (Koolhaas et al., 2010; Veenema and Neumann, 2007). Microinfusion of AVP into the cerebral ventricles (Winslow and Insel, 1993) or within the BNST or lateral septum increases offensive aggression in both hamsters and rats (Delville et al., 1996; Ferris et al., 1984; Irvin et al., 1990; Koolhaas et al., 1998). Denser AVP staining and greater receptor abundance are found in the lateral septum of more aggressive mouse strains (Bester-Meredith et al., 1999). Moreover, deletion of the AVP V1B receptor gene essentially blocks all offensive aggression but does not alter defensive aggression in mice (Wersinger et al., 2002, 2007).

It therefore appears that increased activity in the AVP system in the MeA may be associated with active coping toward threat. One mechanism through which differences in the activity of the MeA AVP system might be maintained is through the methylation status of the AVP promoter. Heightened testosterone results in reduced methylation of the AVP promoter CCGG sequence located at base 2243 (CpG site 1 henceforth) and the CGCG sequence at base 4970 on the AVP promoter (CpG site 2 henceforth), and a subsequent increase in activity in the AVP system (Auger et al., 2011). However, progesterone also plays an important and independent role in regulating AVP expression in the MeA (Auger and De Vries, 2002; Auger and Vanzo, 2006; Bychowski et al., 2013). Specifically, the CGCG sequence at base 4970 on the AVP promoter is in close proximity to progesterone response elements that may facilitate a role for progesterone in regulating the methylation status of the MeA AVP system (Auger et al., 2011; Mohr and Richter, 1990; Shapiro et al., 2000a).

Indeed, progesterone may be of particular relevance in regulating any relationship between coping style and methylation of the AVP promoter that may exist, as different coping styles do not appear to be associated with testosterone (Koolhaas et al., 2010). Conversely, the reduced anxiety and active coping style observed during the estrus phase in female rodents has been linked to lower circulating levels of progesterone (Babar et al., 2008; Gangitano et al., 2009). Another steroid hormone, corticosterone, has also been linked to coping strategy, with lower basal levels of corticosterone associated with an active coping style displayed across a number of tests, such as the resident intruder test and the defensive burying test (Korte et al., 1992, 1996).

Therefore, we examined whether active and passive coping to predator threat was associated with differences in the methylation status of AVP promoters in the MeA and whether these differences are associated with testosterone, progesterone and/or corticosterone levels.

Methods

All experimental procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by the University of Sydney Animal Ethics Committee (approval number L29/7-2010/3/5360).

Subjects

Subjects were 48 adult male Albino Wistar rats (Animal Resources Centre, Perth, Australia), aged 12 weeks at the start of testing. After behavioral screening was completed, it was established that the mean body weight of active ($M = 390.6$, $SEM = 5.1$) and passive ($M = 386.8$, $SEM = 6.6$) responders did not differ ($p > 0.1$). All rats were housed in cages of four with *ad libitum* access to food and water in the home cage and were handled daily for 1 week prior to the start of testing.

Behavior and classification

The threat stimuli used were 2 g balls of cat fur (for more information see S1). Testing was conducted in two identical 1200 mm × 1200 mm × 900 mm ($l \times w \times h$) wooden framed arenas painted matte black, located adjacent to each other (Bowen et al., 2012, 2013; Kendig et al., 2011). The cat fur was placed flush against the center of one of the walls, underneath a wire mesh cylinder (80 mm diameter, 95 mm high, 2.5 mm^2 aperture). The cylinder was also present in the arena during habituation (no fur) sessions in the arena.

The back of each rat was uniquely marked with a Sharpie non-toxic permanent marker to allow identification of individual rats within a quad of four rats. Initially, quads of four rats from the same homecage were placed into the arena for a 50 min habituation session each day for three consecutive days. No cat fur was present during these sessions. Following this, they underwent three further daily test-sessions of 50 min duration with the cat fur present underneath the cylinder.

The number of times each rat made contact with the fur stimulus (defined as a rat placing its nose within 3 cm of the stimulus) during the three fur exposure sessions was used for the classification of rats as active (top 25%), neutral (middle 50%) or passive (bottom 25%). Video recordings of the sessions were made and the number of stimulus contacts made by each rat was scored for the purpose of classification. Classification resulted in 12 passive and 14 active responders. The larger number of active responders was due to 3 rats having the frequency of stimulus contacts corresponding to the 75th percentile.

Plasma and tissue collection

Three days after the final predator odor exposure rats were sacrificed by decapitation without anesthetic and the trunk blood, testicles and brains were collected. All rats were extensively habituated to the procedure leading up to decapitation in the days prior to the sacrifice to ensure minimal stress was induced prior to decapitation. Blood was centrifuged at 4 °C for 15 min at 3300 g, and plasma was collected for analysis. Tissue and plasma were snap frozen in liquid nitrogen and stored at −80 °C.

Methylation of AVP promoter regions in the MePD

The MePD (Bregma −2.76 to −3.24, interaural 6.24 to 5.76; Paxinos and Watson, 2007) was microdissected from snap frozen tissue (for further information on the microdisection procedure please see S1). Extracted gDNA was treated with Methylation Specific Restriction Enzymes (MSRE) *Hpa*II and *Bst*UI (protocol adapted from Auger et al., 2011). The extent of methylation was quantified by qPCR. Briefly, methylated DNA is protected from MSRE digestion, leaving more intact template DNA for the qPCR reaction. Relative expression with reference to no enzyme control was quantified using freely available REST software (http://www.REST.de.com) (Pfaffl et al., 2002). For a more detailed description of this procedure please refer to S1.