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Stereological analyses of reward system nuclei in maternally deprived/separated alcohol drinking rats

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ABSTRACT

The experience of early life stress can trigger complex neurochemical cascades that influence emotional and addictive behaviors later in life in both adolescents and adults. Recent evidence suggests that excessive alcohol drinking and drug-seeking behavior, in general, are co-morbid with depressive-like behavior. Both behaviors are reported in humans exposed to early life adversity, and are prominent features recapitulated in animal models of early life stress (ELS) exposure. Currently, little is known about whether or how ELS modulates reward system nuclei. In this study we use operant conditioning of rats to show that the maternal separation stress (MS) model of ELS consumes up to 3-fold greater quantities of 10% vol/vol EtOH in 1-h, consistently over a 3-week period. This was correlated with a significant 22% reduction in the number of dopaminergic-like neurons in the VTA of naïve MS rats, similar to genetically alcohol-preferring (P) rats which show a 35% reduction in tyrosine hydroxylase (TH)-positive dopaminergic neurons in the VTA. MS rats had a significantly higher 2-fold immobility time in the forced swim test (FST) and reduced sucrose drinking compared to controls, indicative of depressive-like symptomology and anhedonia. Consistent with this finding, stereological analysis revealed that amygdala neurons were 25% greater in number at P70 following MS exposure. Our previous examination of the dentate gyrus of hippocampus, a region involved in encoding emotional memory, revealed fewer dentate gyrus neurons after MS, but we now report this reduction in neurons occurs without effect on the number of astrocytes or length of astrocytic fibers. These data indicate that MS animals exhibit neuroanatomical changes in reward centers similar to those reported for high alcohol drinking rats, but aspects of astrocyte morphometry remained unchanged. These data are of high relevance to understand the breadth of neuronal pathology that ensues in reward loci following ELS.

1. Introduction

The brain's reward system governs natural eating and drinking behaviors that are essential to survival, but this system can be hijacked to mediate the development of substance abuse and addiction (Wise, 2013). The connectivity of and activity within reward loci can be dramatically altered by environmental stimuli during all stages of development. Functionality is particularly susceptible to robust alterations during early developmental stages (Dayan et al., 2010) which can lead to maladaptive behaviors that persist through adulthood. Adverse early life experiences, especially early exposure to chronic stress, was shown to disrupt normal reward seeking mechanisms and confer susceptibility of individuals to neuropsychiatric conditions such as depressive disorders (Enoch, 2011), (Pryce et al., 2005; Ruedi-Bettschen et al., 2006), but especially excessive drug and alcohol use. Increasingly more pre-clinical data has emerged to support the hypothesis that ELS is a risk factor for excessive alcohol use or abuse (Huot et al., 2001; Garcia-Gutierrez et al., 2015). Even in studies where maternal deprivation or separation (MS) does not seem to directly induce increased alcohol preference, evidence suggests that the neuroanatomical or neurochemical disruptions can be dormant until the experience of a second stressor which serves as a trigger for excessive alcohol consumption (Penasco et al., 2015). Thus, the effects of MS are insidious and possibly involve multiple overlapping, sometimes subtle mechanisms.

The basis for this increased predisposition to addictive behavior is not well understood, but early life stress (ELS) can influence the

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microcircuitry of loci that regulate affect, reward, and addiction by modifying neuronal architecture and signaling. In the absence of any drug exposure in ELS animals, corticolimbic structures like the frontal cortex, hippocampus and nucleus accumbens show reduced dendritic length and dendritic spine number following postnatal stress or MS, (Huot et al., 2002; Monroy et al., 2010; Romano-Lopez et al., 2015). Dendritic atrophy was also reported for the PFC (Murmu et al., 2006) and hippocampus (Jia et al., 2010) of prenatally stressed animals. Furthermore, recent data demonstrate heightened expression of GABAA alpha 2 receptors in adult PFC and central amygdala after MS, indicating long-term modulation of brain inhibitory systems (Gondré-Lewis et al., 2016).

In the absence of MS or other ELS, the normal transition from drug overuse to dependency is also characterized by progressive changes in microstructure and circuitry of the brain's reward system (He et al., 2005; Zhou et al., 2007), influencing connectivity, cerebrovascular structure (Cadet et al., 2014), neurochemical substrate levels (Raftogianni et al., 2014), as well as glial cell morphology (Fattore et al., 2002).

The reward system includes the limbic system (hypothalamus, amygdala, hippocampus, septal nuclei and the anterior cingulate gyrus), nucleus accumbens (NAc) and ventral tegmental area (VTA). The VTA, located in the midbrain, contains dopaminergic neurons that project to the NAc and PFC (Aransay et al., 2015) to mediate compulsive drug seeking (Brake et al., 2004), and promotes behaviors associated with the reinforcing effects of ethanol exposure (Gessa et al., 1985; Gatto et al., 1994; Rodd et al., 2005a) and other drugs of abuse. In the NAc of MS rats, there is a reduction in the dopamine transporter DAT presumably as a result of a reduction in afferents from midbrain-localized dopaminergic neurons (Ciliax et al., 1995; Brake et al., 2004), however specific effects of MS on the VTA which has capacity to respond to GABA, glutamate, opioids, cannabinoids, and stress hormones is not known. The VTA can be powerfully regulated by the stress hormone, corticotropin releasing factor (CRF), which when injected into the VTA reinstates drug seeking behavior following extinction of such behavior (Blacktop et al., 2016). Thus stress can directly modify reward and the motivation for drug seeking.

In the limbic system, the amygdala is key for processing emotionally motivated behaviors that are salient in major depressive disorder and addiction (LeDoux, 2007). The amygdala is tightly linked to the dentate gyrus of the hippocampus for processing emotional memory (Abe, 2001; Abe et al., 2008), and indeed, many studies of early life stress, including maternal deprivation/separation stress report extensive alterations in amygdala circuitry (Callaghan and Richardson, 2011; Daniele-wicz and Hess, 2014; Toda et al., 2014). Likewise, alcohol-drinking rats also exhibit depression symptomatology and anxiety, presumably involving amygdala circuits, in the absence of any classic external inducers of these symptoms (Ciccocioppo et al., 2006).

Both the amygdala and the VTA send projections to NAc, where input regarding mood, motivation, and reward converge (LeDoux, 2007; Koob, 2009). The hypothalamic–pituitary–adrenal (HPA) axis, which regulates circulating hormone, is activated in MS to result in heightened expression and release of hormones (ACTH, corticosterone, CRF, other metabolites) from each of its components (Liu et al., 2000; Plotsky et al., 2005; Aisa et al., 2008). These elevated circulating hormones contact receptors throughout the brain but especially on mesocorticolimbic neurons of the VTA, amygdala, and hippocampus (Aisa et al., 2008; Wise and Morales, 2010; Jawahar et al., 2015). In addition, indirect inhibition or activation of receptor systems such as the endocannabinoids and noradrenergic system add additional complexity to the stress response, and could act in parallel, additively or synergistically to amplify a CRF-mediated stress response that facilitates drug consumption and depressive symptoms (Romano-Lopez et al., 2015).

In this study, we hypothesized that adverse events such as early life stress, may alter mesocorticolimbic neuron development and maturation to induce depressive-like behaviors, lack of pleasure seeking, and alcohol drinking. We used a rat model of chronic maternal separation to emulate ELS, in order to investigate the neuroanatomical alterations within reward system nuclei associated with MS-induced alcohol drinking behavior. Because ELS reportedly hijacks the HPA axis, reward circuits, and other neurocognitive features, understanding the altered anatomical correlates to alcohol drinking and other progressive pre-addictive behaviors is essential for the development of effective strategies to combat and prevent addiction.

2. Materials and methods

2.1. Animals

There were a total of 62 animals used in this study. Pregnant Sprague Dawley rats were obtained from Harlan Laboratories (Frederick, MD, USA) and offspring used in this study were born onsite at the veterinary facility. At postnatal day 2, pups were subjected to the MS paradigm described below, and were later behaviorally tested for excessive drinking behaviors as adults. Alcohol preferring (P) and non-alcohol preferring (NP) adult rats were obtained from the Alcohol Research Center, Indiana University School of Medicine. P rats are well characterized as performers of an operant response for access to ethanol that is not performed by the NP rats. Since P rats are genetically predisposed to alcohol drinking and have high CRF levels, they were used in this study to compare the naïve state of their VTA to that of the MS animals to determine if there are similar disruptions in the reward loci of both models. Animals were housed 2 per cage, with a reverse 12 h light/dark cycle and provided with food and water, ad libitum, until operant training after which they were housed 1 per cage. All studies were approved by the Howard University Institutional Animal Care and Use Committee (IACUC), and conducted with strict adherence to the *Guide for the Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011).

2.2. Maternal separation regimen

The maternal separation paradigm was conducted as described previously (Wang and Gondre-Lewis, 2013). Briefly, beginning at P2 until weaning at P21, pups were removed daily from their mothers' home cage, moved to a different room altogether and exposed to maternal separation in individual chambers for 3 h from 11 am to 2 pm each day. The MS room temperature was monitored and maintained at 29 °C with a heater to simulate the warmth of the mother's body. Pups were returned to their mothers' home cage after 3 h. For experimental comparisons, whole litters were assigned to one of two groups: controls (CTL) and maternally deprived/separated (MS). CTL rat pups were kept with their mothers during the entire postnatal period. After MS, rats were weaned at P22 and allowed to mature in the animal facility. They were re-entered into the study in late adolescence (~P42) for the FST, or as adults (>P70) for alcohol responding or immunohistochemistry.

2.3. Operant apparatus and alcohol drinking training protocol

Adult MS rats ($n = 10$) were trained to lever press for ethanol starting at P60-P70. The training described below takes approximately 4 weeks, followed by 21 days of testing. Standard operant chambers (Coulbourn Instruments, Inc., Lehigh Valley, PA) were

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