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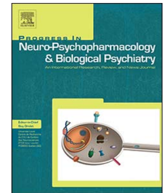

Morphological and functional changes in the preweaning basolateral amygdala induced by early chronic stress associate with anxiety and fear behavior in adult male, but not female rats

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ABSTRACT

Suboptimal maternal care is a form of chronic early-life stress (ELS) and a risk factor for mental illness and behavioral impairments throughout the life span. The amygdala, particularly the basolateral amygdala (BLA), exhibits exquisite sensitivity to ELS and could promote dysregulation of stress reactivity and anxiety-related disorders. While ELS has profound impacts on the adult or adolescent amygdala, less is known regarding the sensitivity of the preweaning BLA to ELS. We employed a naturalistic rodent model of chronic ELS that limits the amount of bedding/nesting material (LB) available to the mother between postnatal day (PND) 1–9 and examined the morphological and functional effects in the preweaning BLA on PND10 and 18–22. BLA neurons displayed dendritic hypertrophy and increased spine numbers in male, but not female, LB pups already by PND10 and BLA volume tended to increase after LB exposure in preweaning rats, suggesting an accelerated and long-lasting recruitment of the amygdala. Morphological changes seen in male LB pups were paralleled with increased evoked synaptic responses recorded from BLA neurons in vitro, suggesting enhanced excitatory inputs to these neurons. Interestingly, morphological and functional changes in the preweaning BLA were not associated with basal hypercorticosteronemia or enhanced stress responsiveness in LB pups, perhaps due to a differential sensitivity of the neuroendocrine stress axis to the effects of LB exposure. Early changes in the synaptic organization and excitability of the neonatal amygdala might contribute to the increased anxiety-like and fear behavior observed in adulthood, specifically in male offspring.

1. Introduction

Optimal mother-infant interactions critically modulate mental and cognitive health in the offspring (Fox et al., 2010; McEwen, 2006) conferring resilience to the development of affective disorders (Franklin et al., 2012), whereas impaired maternal care associated with chronic early-life stress (ELS) (Baram et al., 2012; Essex et al., 2011) increases vulnerability to mood disorders and substance abuse (Weber et al., 2008). Altered regulation in the hypothalamic-pituitary adrenal (HPA) axis in the offspring (Essex et al., 2011) as well as functional changes in several activated brain structures participating in the extended stress circuitry, notably the amygdala, might be implicated in the effects of ELS (Flak et al., 2012; Jankord and Herman, 2008). The basolateral (BLA) and central amygdala (CeA) are involved in the processing and implicit learning of fear and anxiety (LeDoux, 2000; Shin and Liberzon, 2010). As such, they also play a key role in modulating stress responses during exposure to fearful stimuli (Jankord and Herman, 2008; Schulkin, 2006).

The amygdala is extremely sensitive to the effects of both acute (Mitra et al., 2005) and chronic stress exposure (Vyas et al., 2003; Vyas et al., 2006; Vyas et al., 2002). For instance, chronic immobilization stress (CIS) in adult rodents enhances dendrite arborizations of BLA, but not CeA neurons (Vyas et al., 2003; Vyas et al., 2002). CIS also alters neuron morphology in the hippocampus, although in this structure, the changes are reversible, whereas this does not appear to be the case in the BLA (Vyas et al., 2002; Vyas et al., 2004). Functionally, chronic stress in adult rodents also induces BLA neuron hyperexcitability that associates with an anxiety-like phenotype (Rosenkranz et al., 2010).

Exposure to chronic stress during early life and adolescence has lasting effects on the adult amygdala, increasing excitability of BLA pyramidal neurons (Rau et al., 2015) and conditioned fear (Stevenson et al., 2009). However, it is unknown whether similar amygdala

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recruitment already occurs in neonatal rats with the ELS exposure. Recent work demonstrated that global and gene-specific DNA methylation is significantly modified in the amygdala of postnatal day (PND) 30 rats following chronic ELS (Doherty et al., 2016) and adolescent rats exhibited enhanced amygdalar neural activity when exposed to ELS (Raineki et al., 2012). These findings indicate that the consequences of ELS on the amygdala can be observed early, although the proximal effects of ELS on the neonatal amygdala are still unknown. In this study, we used the limited bedding (LB) paradigm as a naturalistic model of ELS (McLaughlin et al., 2016; Molet et al., 2014) to examine neonatal amygdalar function and morphological changes induced by ELS in the preweaning period. We examined the potential contribution of altered neonatal HPA activity in mediating amygdala changes as ELS is known to modify the activity of the HPA axis (Jankord and Herman, 2008) ranging from sensitized to blunted basal and stress responses depending on the life period tested (McLaughlin et al., 2016; Walker et al., 2017). Finally, we determined whether ELS-induced changes in the preweaning amygdala are sex dependent and lead to impairment in behavioral regulation related to anxiety and fear in adult male and female rats.

2. Methods

2.1. Animals

Untimed-pregnant (gestation day 15–16) Sprague-Dawley female rats (Charles River, St-Constant, QC, Canada) were individually housed under controlled conditions of light (12 h light:12 h dark, lights on at 08:00 h), temperature (22–24 °C), and humidity (70–80%) and provided access to rat chow and water ad libitum. The day of birth was considered PND0 and litters were culled to 8–10 pups on PND1. No > 1–2 pups per sex and litter were used for each experimental data point. All experimental procedures were approved by the University Animal Care Committee at McGill University in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Limited bedding paradigm and maternal behavioral observations

The LB paradigm was used between PND1–9 according to a protocol adapted from Baram and colleagues (Molet et al., 2014) with cage changes on PND4 and PND7. On PND1, mothers and their litters were randomly assigned to the limited bedding (LB) or normal bedding (NB) condition. LB mothers and their litters were placed on an aluminum mesh platform 2.5 cm above the cage floor. Approximately 1.5 cm of bedding was added below the platform to cover the cage floor. The dams were given one-half of one paper towel for nesting material. The NB cages received a 2.5 cm layer of woodchips and one-half of one paper towel. On PND10, all LB mothers/litters were returned to NB conditions. All litters were kept with their biological mother for the duration of the experiments. The weights of all pups and dams were recorded on PND1, 4, 7, 9, 14 and 18 and animals were otherwise left undisturbed until experiments and tissue collection on PND10 and PND18–22.

Across all scored experimental cohorts (n = 3, 13 litters/bedding condition), maternal behavior (active/passive nursing, pup grooming, pup retrieving, self-grooming, sleeping, eating, drinking and wandering) is reported between PND5-6 using three 72 min observation periods (two sessions in the light phase and one in the dark phase) as previously described (McLaughlin et al., 2016). Behavior was recorded every minute for each 72 min observation session. The fragmentation of overall behavior, i.e., the degree to which maternal behavior occurs in many short bouts (Baram et al., 2012), during each observation period was determined using a behavioral consistency score in which a score of "1" was given when behavior changed from one epoch (minute) to the next, and "0" when there was no change in the type of behavior exhibited (Ivy et al., 2008).

2.3. Stress procedures

Neuroendocrine responses to stress were compared between NB and LB pups using exposure to 60 min of immobilization (PND10) (McLaughlin et al., 2016) or restraint (PND20). For immobilization stress on PND10, male pups (6–7/group/time point) were placed side-by-side on a plastic surface with two 2 cm-wide strips of adhesive tape placed across the back of each pup while head and limb movements remained unrestricted (McLaughlin et al., 2016). On PND20, pups (6–26/group/time point) were placed in plastic restraint cone bags for 60 min as an acute restraint stress. Pups were sacrificed right before stress onset (0 min) and at the end of stress (60 min) exposure. Trunk blood samples were collected into tubes containing EDTA (60 mg/mL) and plasma was stored at − 20 °C before determination of ACTH and corticosterone (CORT) concentrations. Brains of pups in the no stress (0 min) group were collected, rapidly frozen on dry ice and stored at − 80 °C prior to being sliced for determination of amygdalar volume.

2.4. Preweaning amygdalar volume

Frozen PND10 and 20 brains were sectioned coronally on a cryostat at 18 and 20 μm, respectively, and sections were collected onto Superfrost Plus slides (ThermoFisher, Montreal, QC). The serial sections were dried under vacuum overnight and stored at − 80 °C until stained with Cresyl Violet. The basolateral amygdala (BLA) and central amygdala (CeA) regions were identified by referring to the rat brain atlas of Paxinos and Watson (adult) (Paxinos and Watson, 2007) and Sherwood and Timiras (neonatal) (Sherwood and Timiras, 1970). These structures were contoured on 12 sections/brain for PND10 pups (6 male and female pups/group) and 20 sections/brain for PND20 pups (6 male and female pups/group), using Stereoinvestigator software (Cavalieri method, MicroBrightField, Williston, VT) with the experimenter blind to bedding group. The grid points (20 μm grid spacing) overlapping the contoured areas were counted and converted into volume estimates using the Cavalieri probe after accounting for the non-consecutive section interval and section thickness (Gundersen and Jensen, 1987).

2.5. Morphological analysis of BLA neurons

Naïve NB or LB male and female pups (PND10 or PND20) were anesthetized with ketamine/xylazine (0.1 mL, subcutaneous) and perfused transcardially with 0.9% ice-cold saline-heparin (5USP units/mL heparin) for 5 min. Following perfusion, brains were kept in Golgi-Cox solution (1.04% $K_2Cr_2O_7$, 1.04% $HgCl_2$, and 0.83% K_2CrO_4 diluted in distilled water, all reagents from Fisher Scientific, Fair Lawn, NJ) for 14 days in the dark at room temperature before being transferred in 30% sucrose at 4 °C for 2–7 days (Gibb and Kolb, 1998). Coronal sections (200 μm) were cut using a vibratome (Leica, Concord, ON) and immersed into a 6% sucrose solution. Sections were placed serially onto 2% gelatin-coated slides, dried and stained with 100% ammonium hydroxide followed by fixative (Carestream GBX fixer, diluted 1:1 in distilled water, Sigma-Aldrich, St Louis, MO). Sections were dehydrated in serial alcohol rinses, cleared in xylene and coverslipped. Golgi-Cox stained Class I BLA neurons were selected for analysis on the basis of morphological criteria described previously (McDonald, 1982).

Neurons were manually traced by an experimenter blind to bedding condition, with the Neurolucida software (MicroBrightField, Williston, VT) using a Zeiss Imager M1 microscope with a 100 × objective and a Hamamatsu camera. Digital images were captured with 20 × and 100 × objectives. 1–4 neurons were analyzed per animal (8–10 male and female pups/group), for a total of 21 male and 16–18 female (PND10) and 16 male and 16 female (PND20) neurons per experimental group. Branched structure analyses were performed in Neuroexplorer (MicroBrightField, Williston, VT) on reconstructed neurons to determine total dendritic length, number of branch points and number of dendritic spines. To calculate spine density (spines/μm), the total

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