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Early-life inflammation with LPS delays fear extinction in adult rodents

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ABSTRACT

A large body of evidence has been brought forward connecting developmental immune activation to abnormal fear and anxiety levels. Anxiety disorders have extremely high lifetime prevalence, yet susceptibility factors that contribute to their emergence are poorly understood. In this research we investigated whether an inflammatory insult early in life can alter the response to fear conditioning in adulthood. Fear learning and extinction are important and adaptive behaviors, mediated largely by the amygdala and its interconnectivity with cortico-limbic circuits. Male and female rat pups were given LPS (100 μg/kg i.p.) or saline at postnatal day 14; LPS activated cFos expression in the central amygdala 2.5 h after exposure, but not the basal or lateral nuclei. When tested in adulthood, acquisition of an auditory cued or contextual learned fear memory was largely unaffected as was the extinction of fear to a conditioned context. However, we detected a deficit in auditory fear extinction in male and female rats that experienced early-life inflammation, such that there is a significant delay in fear extinction processes resulting in more sustained fear behaviors in response to a conditioned cue. This response was specific to extinction training and did not persist into extinction recall. The effect could not be explained by differences in pain threshold (unaltered) or in baseline anxiety, which was elevated in adolescent females only and unaltered in adolescent males and adult males and females. This research provides further evidence for the involvement of the immune system during development in the shaping of fear and anxiety related behaviors.

1. Introduction

The extinction of fear is an important learning process; impairments in the extinction process have been associated with disorders such as posttraumatic stress disorder (PTSD) (reviewed in VanElzakker et al., 2014). PTSD is a highly debilitating disorder, with a lifetime prevalence of 1.3–12.3%, depending on geographical location and gender (Breslau, 2009). Even though a high percentage of the population will experience traumatic events throughout their life (between 20 and >80%), only a small percentage (about 10%) will develop ongoing symptoms of fear, stress and anxiety (Breslau, 2009). As such, understanding the biological mechanisms that favor resiliency over vulnerability to developing pathologies following stress has become a significant focus in the research community.

In recent years evidence for an involvement of the immune system has been brought forward that could help explain this variance in susceptibility (reviewed in Michopoulos et al., 2016). In human populations with PTSD, circulating proinflammatory cytokines have been found to be increased, while anti-inflammatory cytokines were unaltered (Gola et al., 2013). Within animal models, severe stressors increase levels of the cytokine interleukin (IL)-1β- which in turn appears to facilitate fear learning- as blocking IL-1β signaling prevents the fear response associated with severe stressors (reviewed in Wang and Young, 2016).

Inflammation during development has a variety of effects on brain and behavior (reviewed by Bilbo and Schwarz, 2012) and has been implicated in a number of pathologies (Green and Nolan, 2014). For example, early-life inflammation affects some aspects of baseline anxiety (Sominsky et al., 2013; Spencer et al., 2005) and alters responses to stressors later in life (Bilbo et al., 2008; Giovanoli et al., 2013; Shanks et al., 1995; Sominsky et al., 2013), decreases social interaction (Doenni et al., 2016; MacRae et al., 2015) and alters some types of hippocampal-dependent fear memory (Bilbo et al., 2006; Harre et al., 2008). While mechanisms that underlie these behavioral changes are not yet clear, there is strong evidence for alterations in neuronal excitability (Galic et al., 2008), neurotransmitters, receptors and transporters (Boissé et al., 2004; Doenni et al., 2016; Harre et al., 2008; Reid et al., 2012; Tenk et al., 2007; Zavitsanou et al., 2013) as well as peripheral hormones and autonomic responses associated with

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arousal (Kentner et al., 2010; Sominsky et al., 2013). It is thus possible that immune activation early in life predestines an individual to susceptibility for anxiety disorders.

An important brain structure that is known to be affected in anxiety disorders is the amygdala (reviewed by Weston, 2014). The amygdala is one of the brain's major control centers of affective states and influences both emotion and motivation (reviewed by Janak and Tye, 2015) and is essential for the acquisition and extinction of cued fear memory (Phillips and LeDoux, 1992). Interestingly, in adult rodents the amygdala responds to inflammation with neuronal activation, which has been shown by increased FosB/ΔFosB and cFos expression (Frenois et al., 2007; Prager et al., 2013) as well as intracerebral encephalography (Prager et al., 2013). Permanent alterations in amygdaloid circuits by neuronal changes induced by developmental inflammation could increase the susceptibility to pathologically express aberrant behaviors.

In rodent development, around postnatal day (P) 14, the amygdala is developing extensive connections (Verwer et al., 1996), which makes P14 an extremely interesting temporal window when looking at early immune activation and its consequences. Inflammation on P14 causes an increase in proinflammatory cytokines in the adult amygdala, while anti-inflammatory cytokines remain largely unaffected (Dinel et al., 2014). Neurons are particularly vulnerable to cytokine exposure during development (reviewed by Borsini et al., 2015), thus raising the possibility of inflammation-induced alterations in amygdala development. We have previously shown early inflammation-induced alterations in specific amygdala-related social behaviors in adolescent animals (Doenni et al., 2016) leading us to hypothesize that changes may also persist into adulthood and affect other amygdala functions. Furthermore, it has previously been shown that LPS acutely affects fear extinction of an auditory cued fear response, but not contextual fear, furthering the idea that inflammation may influence amygdala function in a very specific manner (Quiñones et al., 2016). A similar effect has been shown for interferon-α, which delays auditory cued fear extinction when injected directly into the basolateral amygdala (BLA) (Bi et al., 2016).

On basis of the literature mentioned above we hypothesized that inflammation with LPS on P14 acutely activates the amygdala and alters amygdala function such that it changes aspects of learning and/or extinction of cued fear memory, but not contextual memory in adult rats. We further tested for differences in anxiety-like behavior, as it has been suggested to correlate with altered or impaired fear extinction (Gazendam et al., 2013) and adolescent (Kiliç et al., 2008) as well as adult (Christiansen and Elklit, 2008) anxiety levels have been suggested to be predictors for PTSD in human populations. In light of the fact that much of the previous literature addressed the impact of early life inflammation on males only, we asked if females and males would respond similarly.

2. Materials and methods

2.1. Animals

Sprague Dawley (SD) rats (Charles River Laboratories) were maintained under specific pathogen free conditions and were bred in-house to avoid transport stress during pregnancy. For breeding, females were placed with a male for 7 days, after which males were removed and females completed pregnancy and rearing in single housed conditions. Litters were culled between P3 and P7 to 12 pups (~50% male and female). No more than two pups per litter/gender, per treatment group were used for any behavioral measure. Separate cohorts of animals were used for each experiment to prevent confounding effects of the experimental procedures. Anxiety testing was executed at P40-45, whereas fear conditioning and extinction and additional anxiety testing was performed in young adults (P60-65). Thermal sensitivity was examined in adult rats (P60-90). Neuronal activation was quantified in P14 rat pups. For an overview of experiments see 1A.

2.2. Early immune activation

Animals were assigned to either treatment or control group (~50% split for both males and females) and were injected on P14 (between 1100 h and 1300 h) with LPS (*Escherichia coli*, serotype O26: 100 μg/kg; i.p.) or pyrogen-free saline. Ears were notched to mark treatment. On P21 rats were weaned and housed in groups of 2–3 in clear Plexiglas cages (43 × 21 × 22 cm) with wood chip bedding. Each cage contained a minimum of 1 LPS and 1 saline injected animal. Animals were maintained under 12:12 h illumination cycle (lights on 0700 h) at room temperature (20–22 °C), and received *ad lib* access to lab chow and water. Experimental procedures were approved by the University of Calgary Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.3. cFos activation

Animals (P14, n = 39; 19 LPS/20 Saline, 18 male/21 female; 4 litters) were removed from the dam and injected with 100 μg/kg LPS or saline as described in Section 2.2. A waterproof marker was used instead of the ear notches to mark treatment to avoid any interference of injury and/or pain with the effect of inflammation. Pups were returned to the dam for 2.5 h. Subsequently, pups were anesthetized with isoflorane and perfused through the heart with 10 ml chilled phosphate buffered saline (PBS) (pH = 7.2) and then fixed with 5 ml 4% paraformaldehyde (PFA). Brains were extracted, post-fixed overnight in 4% PFA at 4 °C and then stored in cold PBS containing 20% sucrose until sectioning at 40 μm on a cryostat. Slices were stored in PBS containing 0.05% sodium azide (Sigma Aldrich).

The free-floating slices were stained in net wells. Briefly, the tissue was washed with PBS and subsequently incubated with 0.3% hydrogen peroxide for 30 min. After additional washing steps, free binding sites were blocked by 60-min incubation in blocking solution (10% goat serum, 1% triton X in PBS). CFos antibody (SC-52, 1:1000, Santa Cruz) was incubated overnight at room temperature in T-PBS containing 1% goat serum. After three additional washing steps, the secondary antibody (Biotinylated goat anti rabbit, 1:500, Vector, USA) was incubated for 1 h. Subsequently, the ABC kit (VECTASTAIN®Elite ABC, Vector, USA) was applied according to manufacturer's instructions, preceded and followed by each three PBS washes. DAB peroxidase substrate kit (Vector, USA) was applied for 15 s and, after additional washes, slices were mounted on positively charged glass slides. Slices were dried and cleaned by treatment with alcohol and xylene and coverslipped with Permount© (Fisher Scientific, USA).

Amygdala slices were sorted for their location from anterior to posterior and digital images of similar coronal anterior-posterior sections of basal (BA), lateral (LA) and central (CeA) amygdala were chosen for experimental and control groups. Each animal was assessed bilaterally in two separate sections, spaced by approximately 700 μm. Similar regions of interest in each nucleus were assigned by an evaluator blinded for treatment. Grey values of the background staining were automatically assessed by ImageJ and the same amount of background was subtracted from each individual picture. Cells were then counted automatically, where 50 or more pixels were defined as a cell within BA and LA (large cortex-like pyramidal neurons) and 35 or more pixels were