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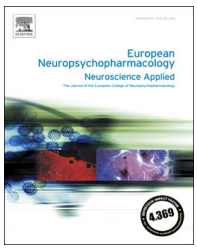

Neonatal handling enduringly decreases anxiety and stress responses and reduces hippocampus and amygdala volume in a genetic model of differential anxiety: Behavioral-volumetric associations in the Roman rat strains

Cristóbal Río-Álamos[a,*], Ignasi Oliveras[a], Maria Antonietta Piludu[b], Cristina Gerbolés[a,b,c,d], Toni Cañete[a], Gloria Blázquez[a], Silvia Lope-Piedrafita[c,d], Esther Martínez-Membrives[a], Rafael Torrubia[a], Adolf Tobeña[a], Alberto Fernández-Teruel[a,*]

[a]*Department of Psychiatry & Forensic Medicine, Institute of Neurosciences, Autonomous University of Barcelona, 08193-Bellaterra, Barcelona, Spain*
[b]*Department of Life and Environmental Sciences, Section of Pharmaceutical, Pharmacological and Nutraceutical Sciences, University of Cagliari, 09124 Cagliari, Italy*
[c]*Servei de RMN, Autonomous University of Barcelona, 08193-Bellaterra, Barcelona, Spain*
[d]*Centro de investigacion Biomédica en Red – Bioingenieria, Biomateriales y Nanomedicina (CIBER-BBN), Spain*

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Abstract
The hippocampus and amygdala have been proposed as key neural structures related to anxiety. A more active hippocampus/amygdala system has been related to greater anxious responses in situations involving conflict/novelty. The Roman Low- (RLA) and High-avoidance (RHA) rat lines/strains constitute a genetic model of differential anxiety. Relative to RHA rats, RLA rats exhibit enhanced anxiety/fearfulness, augmented hippocampal/amygdala c-Fos expression following exposure to novelty/conflict, increased hippocampal neuronal density and higher endocrine responses to stress. Neonatal handling (NH) is an environmental treatment with long-lasting anxiety/stress-reducing effects in rodents. Since hippocampus and amygdala volume are supposed to be related to anxiety/fear, we hypothesized a greater volume of both areas in RLA than in RHA rats, as well as that NH treatment would reduce anxiety and the volume of both

*Corresponding authors.
E-mail addresses: cristobal.delrio@uab.cat (C. Río-Álamos), Albert.Fernandez.Teruel@uab.cat (A. Fernández-Teruel).

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structures, in particular in the RLA strain. Adult untreated and NH-treated RHA and RLA rats were tested for anxiety, sensorimotor gating (PPI), stress-induced corticosterone and prolactin responses, two-way active avoidance acquisition and *in vivo* 7 T 1H-Magnetic resonance image. As expected, untreated RLA rats showed higher anxiety and post-stress hormone responses, as well as greater hippocampus and amygdala volumes than untreated RHA rats. NH decreased anxiety/stress responses, especially in RLA rats, and significantly reduced hippocampus and amygdala volumes in this strain. Dorsal striatum volume was not different between the strains nor it was affected by NH. Finally, there were positive associations (as shown by correlations, factor analysis and multiple regression) between anxiety and PPI and hippocampus/amygdala volumes.

1. Introduction

Previous studies have shown positive associations between anxiety-related temperament/responses and hippocampus and amygdala activity/connectivity and volume in healthy humans (Barros-Loscertales et al., 2006; Cherbuin et al., 2008; Hahn et al., 2010; Levita et al., 2014), as well as between anxious temperament and activation of both structures in rhesus monkeys (Oler et al., 2010). Remarkably, a causal role for the hippocampus in solving (anxiety-mediated) approach-avoidance conflicts has been shown by both positive associations between hippocampal activity and behavioural inhibition in healthy volunteers, and by passive avoidance deficits in hippocampus-lesioned patients (Bach et al., 2014).

The septo-hippocampal system, in close interplay with the amygdala, are key neural mechanisms in the regulation of anxiety as a crucial part of the behavioral inhibition system (Davis and Whalen, 2001; Gray and McNaughton, 2000; LeDoux 2000). Gray's theory has recruited wide support from animal lesion and pharmacological studies, confirming that the more active the hippocampus and amygdala the greater the anxious/fearful responses (Gray and McNaughton, 2000; Mc Naughton, Corr, 2004), but results on associations between anxiety-hippocampus volume in untreated rats and in rat lines selected for divergent anxiety have been inconclusive (Kalisch et al., 2006).

A well-characterized model of anxiety are the Roman High- (RHA) and Low-avoidance (RLA) rat lines/strains, psychogenetically selected for their good (RHA) vs poor (RLA) acquisition of the two-way active avoidance response, a "passive avoidance/active avoidance" conflict that involves anxiety (e.g. Driscoll et al., 1998, 2009; Escorihuela et al. 1999; Fernández-Teruel et al., 1991; Gray and McNaughton, 2000). Compared with RHAs, RLA rats display increased anxiety/stress-related responses (e.g., Carrasco. et al., 2008; Díaz-Morán et al., 2012; Estanislau et al., 2013; López-Aumatell et al., (2009a, 2009b), decreased GABA-A receptor function (Bentareha et al., 1998; Giorgi et al., 1994), enhanced anti-anxiety effects of anxiolytic substances/treatments (Martin et al., 1982; Steimer and Driscoll 2003; Torres et al., 2007) and a more functional/active hippocampus (Garcia-Falgueras et al., 2012; Meyza et al., 2009). Hippocampal volume is supposed to reflect its overall activity (see discussion in e.g. Barros-Loscertales et al., 2006; Kalisch et al., 2006), thus we would expect increased hippocampal volume in RLAs as compared with RHA rats.

An environmental treatment with well-known long-lasting anxiolytic effects is neonatal handling (NH), which enduringly reduces anxiety/stress responses in a variety of tests/tasks in rodents, including RLA/RHA rats (e.g. Anisman et al., 1998; Fernández-Teruel et al., 1997, 2002; Meaney et al., 1988; Steimer et al., 1998; Raineki et al., 2014). As the hippocampus and amygdala are crucial in the regulation of anxiety, we expected that an enduring reduction of anxiety in NH-treated RLA rats would be paralleled by a decrease in volume of these structures. We also expected no changes in the dorsal striatum, which we have used as a "control" brain region not related with anxiety. In separate batches of control and NH-treated animals we have also evaluated stress hormone responses (corticosterone, prolactin) and two-way avoidance acquisition (as an index of anxiety; see Fernández-Teruel et al., 1991; Gray and McNaughton, 2000). Post-stress levels of corticosterone and prolactin were assessed as independent markers of stress sensitivity and of NH effects, because both hormonal responses are known to reflect the levels of perceived stress and, prolactin, in particular, has even been related to the levels of anxiety in rats (for review see Díaz-Morán et al., 2012, and references therein).

2. Experimental procedures

2.1. Animals

Pregnant inbred Roman High- (RHA, $n=16$) and Low-Avoidance (RLA, $n=17$) female rats (Autonomous University of Barcelona), from the 60th inbreeding generation were used. They were individually housed and were maintained with food and water freely available, 12-h light-dark cycle (light on at 0800 h) and controlled temperature (22 ± 2 °C). They were randomly distributed across the following four experimental groups to which their offspring would be assigned: RHA and RLA control (C), nonhandled rats, and RHA and RLA animals that received neonatal handling (NH) treatment. After weaning (postnatal day 21st) the pups were housed in pairs of the same experimental group in standard macrolon cages ($50\times25\times14$ cm^3).

For the first experiment (Batch 1) 32 RHA and 32 RLA male rats (16 in each of the 4 experimental groups) were used for behavioral evaluation between 2 and 4 months of age (see below), and for structural MRI assessment at 6 months of age. Each of the 4 experimental groups was composed of 16 male rats representing

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