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Research Paper

The habenula as a critical node in chronic stress-related anxiety

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ABSTRACT

The habenula is activated in response to stressful and aversive events, resulting in exploratory inhibition. Although possible mechanisms for habenula activation have been proposed, the effects of chronic stress on the habenular structure have never been studied. Herein, we assessed changes in volume, cell density and dendritic structure of habenular cells after chronic stress exposure using stereological and 3D morphological analysis. This study shows for the first time that there is a hemispherical asymmetry in the medial habenula (MHb) of the adult rat, with the right MHb containing more neurons than its left counterpart. Additionally, it shows that chronic stress induces a bilateral atrophy of both the MHb and the lateral habenula (LHb). This atrophy was accompanied by a reduction of the number of neurons in the right MHb and the number of glial cells in the bilateral LHb, but not by changes in the dendritic arbors of multipolar neurons. Importantly, these structural changes were correlated with elevated levels of serum corticosterone and increased anxious-like behavior in stressed animals. To further assess the role of the habenula in stress-related anxiety, bilateral lesions of the LHb were performed; interestingly, in lesioned animals the chronic stress protocol did not trigger increases in circulating corticosterone or anxious-like behavior. This study highlights the role of the habenula in the stress responses and how its subregions are structurally impacted by chronic stress with physiological and behavioral consequences.

1. Introduction

The habenula is a small bilateral brain region that together with the pineal gland forms the epithalamus and is phylogenetically preserved in almost all vertebrates (Andres et al., 1999; Stephenson-Jones et al., 2012). The habenular nuclei, which consist of the medial and lateral sub-regions (MHb and LHb, respectively), have been described as a relay interface between the basal ganglia and the limbic system since forebrain projections from both converge there (Herkenham and Nauta, 1977) and are able to modulate downstream limbic midbrain dopamine and serotonin circuits (Hikosaka et al., 2008; Hikosaka 2010; Stephenson-Jones et al., 2012; Zhao et al., 2015). This modulation can both promote or inhibit release of dopamine and serotonin in the brain since the habenula is strongly connected with the ventral tegmental area (VTA) – via the rostromedial tegmental nucleus (rMTG) – and the raphe nucleus (RN) – via the interpeduncular nucleus (IPN) (Christoph et al., 1986; Ferraro et al., 1996; Lecourtier et al., 2008; Zhao et al., 2015) –, the major sources of dopaminergic and serotonergic projections in the brain, respectively (Vertes, 1991; Russo and Nestler, 2013). Although the LHb appears to be the only habenula sub-region capable of directly controlling dopamine release, serotonergic modulation is likely more complex as both structures exert influence on the IPN – the MHb directly and the LHb indirectly – and the RN (Hikosaka et al., 2008; Hikosaka, 2010; Stephenson-Jones et al., 2012; Zhao et al., 2015), suggesting that the combined output of both structures is particularly critical for the serotonergic system. Moreover, the MHb also projects to the LHb (Kim and Chang, 2005) and both sub-structures share several efferent inputs (Herkenham and Nauta, 1977) further highlighting the complexity of the combined net output of the habenula This critical modulatory role of the monoamines circuits has been linked to several different behaviors and conditions (Hikosaka, 2010; Fakhoury and López, 2014; Proulx et al., 2014), therefore attracting increased attention from researchers focusing on mood disorders, depression and stress (Hikosaka, 2010).

The particular involvement of the habenula in the stress response was initially attributed to the observation that habenular neurons were activated by a variety of stressors (Wirtshafterm et al., 1994) and aversive/unpleasant stimuli including both the presence of punishment and the absence of reward (Matsumoto and Hikosak, 2009). Additionally, stimulation of the habenula produced autonomic responses similar to those observed in emotional stress (Ootsuka and Mohammed, 2015) and LHb activity was correlated with behavioral inhibition in normal and stressful contexts (Lee and Huang, 1988). This inhibitory role has been linked to the modulation of dopamine and serotonin release in the brain and has been additionally shown in several lesion (Murphy et al., 1996; Yang et al., 2008; Tian and Uchida, 2015),

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excitation (Christoph et al., 1986; Ji and Shepard, 2007) and inhibition (Winter et al., 2011; Sachs et al., 2015) and optogenetic (Stamatakis and Stuber, 2012; Amo et al., 2014) studies.

Chronic exposure to stress leads to monoamines dysfunction with behavioral consequences (Chaouloff et al., 1999; Pani et al., 2000; Bambico et al., 2009) that mimic those described for overactivation of the habenula. Moreover, several depression studies that use chronic stress exposure as a model have shown an involvement of the habenula in the dysregulation of the monoamines circuits with functional and behavioral consequences (Christensen et al., 2013; Aizawa et al., 2013; Proulx et al., 2014). More strikingly, it was also shown that habenula-induced monoamine dysfunction could be one of the causes for increased susceptibility to stress (Sachs et al., 2015). Yet, and although some mechanisms for habenula overactivation have been proposed (Aizawa et al., 2013; Li et al., 2013; Cui et al., 2014), the consequences of chronic exposure to stress on habenular structure have never been explored.

Here, we assessed how chronic exposure to stress could lead to changes in volume, cell density and dendritic structure of habenular cells by stereological and 3D morphological analysis of Giemsa and Golgi stained LHb and MHb sections. We not only present, for the first time, an unbiased estimation of the total number of neuronal cells in the MHb and LHb and glial cells in the LHb of the adult rat in both brain hemispheres, but also how chronic stress can disrupt the basal structure. Anxiety-related disorders - despite of their clinical importance – have received far less attention than depression in previous habenula studies; therefore, we additionally assessed if habenular integrity was necessary for the physiological and behavioral expression of stress-related anxiety by exposing habenula-lesioned animals to a chronic stress protocol and then to an anxiogenic environment. Considering it had been shown that lesions of the LHb after a stress protocol could alleviate some of the immediate behavioral and physiological symptoms of depressive-like behavior (Yang et al., 2008) we also hypothesized that ablation of the LHb previous to the stress exposure could block the escalation of stress-related anxiety symptoms due to stress exposure.

2. Materials and methods

2.1. Animals

A total of 36 Male Wistar-Han rats (Charles River laboratories, Barcelona, Spain), weighting 300-400 g and aged 12–16 weeks were used in this study. Animals were pair-housed under the following laboratory conditions: room temperature of 22 °C, relative humidity of 55%, 12 h light cycle beginning at 8 AM and food and water *ad libitum*. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Experiments were conducted in accordance with European Union Directive 2010/63/EU and the Portuguese regulations and laws on the protection of animals used for scientific purposes of the Ministry for Agriculture, Rural Development and Fishing. This study was approved by the Portuguese Veterinary General Direction, Direção Geral de Alimentação e Veterinária (DGAV).

Animals were divided into 4 groups: control (n = 12), stress (n = 12), lesion-control (n = 6) and lesion-stress (n = 6).

2.2. Surgery/electrolytic lesion

Animals from the lesion (lesion-control and lesion-stress) groups were subjected to bilateral electrolytic ablation of the LHb by passing a current through a concentric tungsten electrode (PI2CEA10, Microprobes, Gaithersburg, MD, USA). An adaptation of the lesion protocol of (Haack et al., 2014) was used: since the habenula has a quasi-tubular form, anterior and posterior lesions were performed in order to increase the extent of the lesion in the targeted area and reduce the damage to neighboring regions. Coordinates for the sites were: −3.0 and −3.7 mm AP, ±0.8 mm ML, and −5.4 mm DV, from bregma. Lesions were produce by passing a current of 1 mA for 6 and 10s in the most anterior and most posterior sites, respectively. Lesions were confirmed in Cresyl-violet stained 30 μm slices from the *post-mortem* brains. Supplementary Fig. 1 shows a schematic representation of the average lesion extent on all lesioned animals. Although the electrolytic lesions were largely confined to the LHb, in some sites the lesion extended beyond the LHb to surrounding structures, including the MHb. Three rats (2 from the lesion-control group and 1 from the lesion-stress group) were removed from further data analysis due to poorly targeted lesions.

Animals were allowed to recover from surgery for 1–2 weeks.

2.3. Stress protocol

Rats from the stress groups (stress, and lesion-stress) were then exposed to a chronic unpredictable stress (CUS) protocol, described elsewhere (Cerqueira et al., 2007), for 28 days. Importantly, exposure to this CUS protocol is known to induce anxiety-like behavior (Pêgo et al., 2008; Jacinto et al., 2013). Briefly, stressed animals were exposed to a daily stressor (up to 1 h a day). In order to avoid adaptation the stressor applied was different every day and presented at a different and random hour of the day. Five different stressors were used: restraint, noise, shaking, cold water and hot air stream. All stressors were applied in a separate experimental room from where the animals of both groups were housed. Control group animals (control and lesion-control) were handled for the same time during the same period.

On the day following the end of the stress protocol blood samples were drawn from all animals (from the 4 groups) via tail venipuncture for serum corticosterone levels assessment. Blood samples were collected in the morning (8 AM). The samples were centrifuged at 13000 rpm for 10 min. Serum was extracted and stored at −80 °C for posterior analysis. Serum corticosterone levels were measured by immunoassay using a commercially available ELISA kit (Corticosterone ELISA kit, Enzo Life Sciences, Farmingdale, NY, USA).

2.4. Behavior

Following one day of rest after blood collection, animals from all groups were exposed to the Elevated-Plus Maze (EPM) test for 5 min. The EPM is a validated test to assess anxiety-like behavior in rodents and the protocol has been described elsewhere (Sousa et al., 2006). Briefly, the test apparatus consists of an elevated plus shape where two of the plus' arms are surrounded by high walls and the two other are not. The test explores the conflict of appetitive and aversive motivations to explore novel environments and avoid brightly lit and elevated spaces, respectively, Responsiveness to the anxiety-inducing environment was measured as the time animals spent exploring the open arms of the EPM, regarded as most aversive areas of the test. Animals which present higher anxiety-levels (or higher response to anxiety-inducing stimuli) tend to spend less time exploring the open arms (Pêgo et al., 2008). Since the EPM was performed at different times across different groups and subtle environmental variations may impact on behavior – even though the order of the animals was randomized –, the time spent in the open arms was transformed in a z-score when all groups were compared. The averaged non-z-transformed data is also shown as Supplementary Fig. 2.

2.5. Stereology

Stereological procedures were similar to the ones described in (Cerqueira et al., 2007). On the day following the behavioral testing part of the rats from the control (n = 6) and stress (n = 6) groups were anaesthetized with an overdose of pentobarbital injected intraperitoneally and perfused transcardially with a fresh solution of 0.9% saline followed by a fixative solution (4% paraformaldehyde). The brains were removed, cut in a 5 mm section that included all of the habenula, with