EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Sexually Dimorphic Changes of Hypocretin (Orexin) in Depression

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ARTICLE INFO

Article history:
Received 14 November 2016
Received in revised form 27 March 2017
Accepted 27 March 2017
Available online xxxx

Keywords:
Depression
Hypocretin
Hypocretin receptors
Hypothalamus
Sex difference

ABSTRACT

Background: Neurophysiological and behavioral processes regulated by hypocretin (orexin) are severely affected in depression. However, alterations in hypocretin have so far not been studied in the human brain. We explored the hypocretin system changes in the hypothalamus and cortex in depression from male and female subjects.
Methods: We quantified the differences between depression patients and well-matched controls, in terms of hypothalamic hypocretin-1 immunoreactivity (ir) and hypocretin receptors (Hcrtr-receptors)-mRNA in the anterior cingulate cortex (ACC) and dorsolateral prefrontal cortex. In addition, we determined the alterations in the hypocretin system in a frequently used model for depression, the chronic unpredictable mild stress (CUMS) rat.
Results: i) Compared to control subjects, the amount of hypocretin-immunoreactivity (ir) was significantly increased in female but not in male depression patients; ii) hypothalamic hypocretin-ir showed a clear diurnal fluctuation, which was absent in depression; iii) male depressive patients who had committed suicide showed significantly increased ACC Hcrt-receptor-2-mRNA expression compared to male controls; and iv) female but not male CUMS rats showed a highly significant positive correlation between the mRNA levels of corticotropin-releasing hormone and prepro-hypocretin in the hypothalamus, and a significantly increased Hcrt-receptor-1-mRNA expression in the frontal cortex compared to female control rats.
Conclusions: The clear sex-related change found in the hypothalamic hypocretin-1-ir in depression should be taken into account in the development of hypocretin-targeted therapeutic strategies.
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1. Introduction

In individuals vulnerable to depression and suicide an exaggerated reaction to environmental stressors, such as life events, is found in the stress-regulating systems of the brain (Turecki et al., 2012). This hyper-reactivity stems from the interaction of genetic, early developmental and environmental factors (Sun et al., 2013). The hypothalamo-pituitary-adrenal (HPA) axis holds a prominent position in the network mediated by stress- and reward-related neurotransmitters and neuromodulators, and is significantly affected in depression (Bao et al., 2012) and suicide (Turecki et al., 2012).In depression, the hypothalamic paraventricular nucleus (PVN), which is the regulating center of the HPA-axis, shows not only an increased production of corticotropin-releasing hormone (CRH) (Raadsheer et al., 1995; Bao et al., 2005), but also changes in the expression of certain CRH-activity-related receptors that render the PVN more sensitive to stress (Wang et al., 2008). Both increased CRH and increased corticosteroid levels may induce depressive-like behavioral changes (Holsboer, 2001).

Hypocretin-producing neurons are localized in the hypothalamus (Hunt et al., 2015) and act via their two G-protein-coupled receptors, hypocretin receptor-1 and -2 (Hcrt-receptor-1 and Hcrt-receptor-2) (Sakurai et al., 1998). Hypocretin projections and Hcrt-receptors are found in many brain areas, including the prefrontal cortex (PFC) (Peyron et al., 1998). It is of interest to note that the neurophysiological and behavioral processes regulated by hypocretin, such as the sleep-wake cycle, food intake, sexual behavior, and stress response, are severely affected in depression (Nollet and Leman, 2013). Several studies have indicated the possible involvement of the hypocretin system in depression with suicide. For example, reduced CSF hypocretin levels were observed in suicidal patients with major depressive disorder (MDD) compared with patients suffering from adjustment disorder or dysthymia with suicide attempts (Brundin et al., 2007a). In addition, significant negative correlations were found between CSF-hypocretin levels and the symptoms of lassitude (difficulty to initiate activities) and slowness of movement, as well as the ratings of global illness (Brundin et al., 2007b). Hypocretin has close functional interactions with the HPA-axis.

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http://dx.doi.org/10.1016/j.ebiom.2017.03.043
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Please cite this article as: Lu, J., et al., Sexually Dimorphic Changes of Hypocretin (Orexin) in Depression, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.03.043

In rat, CRH directly stimulates the release of hypocretin during acute stress (Winsky-Sommerer et al., 2004). Hcrt-receptor antagonists were found to attenuate anxiety and panic-like behaviors associated with stress or hyperarousal states in rat (Johnson et al., 2015; Bonaventure et al., 2015). Moreover, the changes in the transcripts for the Hcrt-receptor-1 and Hcrt-receptor-2 were found to be divergent, i.e. Hcrt-receptor-1 increased, while Hcrt-receptor-2 decreased in the basolateral amygdala in chronically stressed c57bl/6 mice (Arendt et al., 2014). A clinical study of single-nucleotide polymorphisms suggested that the hcrt-receptor-1 gene, or a linked locus, may modulate the risk for mood disorders (Rainero et al., 2011). This possibility was confirmed in hcrt-receptor-1 knockout mice, which showed increased anxiety-like behavior and altered depression-like behaviors (Abbas et al., 2015). These data indicate that the hypocretin system may have a bi-directional regulatory capacity in terms of the stress response.

In light of the data mentioned above, we hypothesized that the hypocretin/orexin system may play a role in the pathogenesis of depression and suicide, possibly by interaction with the HPA-axis. To test this possibility, we measured hypocretin-1 expression in its production area, the postmortem hypothalamus. Second, Hcrt-receptors-mRNA content was determined in the PFC of depressive patients, some of whom had committed suicide. Finally, we determined prepro-hypocretin-mRNA and CRH-mRNA in the hypothalamus and Hcrt-receptors-mRNA levels in the frontal cortex in a frequently used animal model for depression, i.e. chronic unpredictable mild stress (CUMS) rats. Because of the clear sex differences in depression and suicide, special attention was given to the possible sex differences, both in the human and in the animal studies (Bao and Swaab, 2011).

2. Materials and Methods

2.1. Part I: Post-mortem Brain Material Study

In total, 120 human post-mortem samples were studied: 32 hypothalami and 52 cortex samples from the Netherlands Brain Bank (NBB), and 36 cortex samples from the Stanley Medical Research Institute (SMRI). Informed consent for a brain autopsy and for the use of the brain material and medical records for research purposes was given by the donor or their next of kin.

The chronic mood disorder patients and their controls were well-matched for confounding factors, including age, sex, postmortem delay, fixation time, clock time of death, month of death, CSF-pH (a measure of agonal state), brain weight and Braak stages of Alzheimer's pathology (Braak and Braak, 1991). Clinico-pathological details and p-values of matching are given in Table 1 and Supplementary Tables 1–2. The diagnosis of MDD or bipolar disorder (BD) was confirmed according to the Diagnostic and Statistical Manual of Mental Disorders IV by qualified psychiatrists using the extensive medical records of the NBB, which also contained well-documented diagnoses and onset of depression from psychiatric clinics. Exclusion criteria for control subjects were in the first place the use of corticosteroids, as they inhibit the CRH cells in the human hypothalamus (Watts, 2005), which may subsequently influence the hypocretin system (Brunton and Russell, 2003). In addition, primary neurological or psychiatric diseases were exclusion criteria, unless stated otherwise. The absence of pathology was verified in all subjects by a systematic neuropathological analysis (Fronczek et al., 2007; Gao et al., 2013).

2.1.1. Immunocytochemistry and Quantification

The hypothalami were fixed in 10% PBS (pH 7.4) formalin at room temperature and were paraffin-embedded and serially-sectioned at 6 μm in rostro-caudal direction. Hypothalami of 16 mood disorder patients, i.e. 9 MDD and 7 BD patients, and 16 control subjects were used. Every 100th section of 6 μm thickness in the expected hypocretin cell area was stained using a hypocretin-1 antibody (catalog no. H-003-30, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) at 1:20,000 dilution. The specificity of the antibody had been confirmed in our previous study (Fronczek et al., 2007). In addition, we stained the hypothalamus of a narcoleptic patient as a negative control whose hypothalamus was indeed virtually devoid of hypocretin-1-immunoreactive (ir) neurons.

Hypocretin-1-ir was quantified by the image analysis procedure described in our previous studies (Gao et al., 2013). In brief, the set-up consisted of an image analysis system (Image Proversion 6.3, Media Cybernetics, Rockville, USA) connected to a black and white camera (SONY XC-77E) mounted on a microscope (Zeis Axios-kop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). The hypothalamus area covered by hypocretin-stained neurons was outlined manually at a 20× objective. The threshold for the positive signal was set at twice the optical density (OD) of the background. The computer determined the OD of the pixels and percentage surface area covered by the signal (area mask). The integrated optical density (IOD) was calculated by multiplying the OD by the masked area corrected for background. For each subject, the total IOD was calculated as the final parameter for the total amount of Hypocretin-1-ir, by a conversion program based upon multiplication of the separate IOD by sample frequency of the sections, as described previously (Goldstone et al., 2002). Completeness of the hypocretin-1-ir in the hypothalamus was confirmed by graphically presenting the IODs measured in every section from rostral to caudal, with a line drawn by the excel trend-line option 'Moving Average'. The value under each curve showed the total IOD of the subject (see Fig. 1A–B). The total IOD of hypothalamic hypocretin-1-ir as determined by computer-assisted morphometry is not only an objective method but also less time-consuming than neuron-counting. In addition, the IOD shows a good positive correlation with neuron-counting (for 35 sections of 2 patients and 2 controls, rho = 0.781, $p < 0.001$), performed in the way we described previously (Fronczek et al., 2007).

2.1.2. Quantitative PCR (qPCR) for mRNA Expression of Hcrt-receptors

In our present study 50 μm cryostat sections of anterior cingulate cortex (ACC) or dorsal lateral prefrontal cortex (DLPFC) were cut from the left side of the cortex and the grey matter containing all six layers was isolated as described before (Gao et al., 2013). Levels of Hcrt-receptor-1- and Hcrt-receptor-2-mRNA were determined by qPCR. Information regarding gene selection and primers are shown in Supplementary Table 3. RNA isolation, cDNA synthesis and qPCR were performed as described before (Wang et al., 2008).

In addition, a normalization strategy was used to remove sampling-related differences in RNA quantity (Wang et al., 2008). The expression of target genes was normalized using the sets of stable reference genes, including actin-beta (ACTb), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ubiquitin-C for the postmortem human brain study, while ACTb, GAPDH, HPRT1, and elongation factor-1 alpha (EF1α) for the animal study (see below).

2.2. Part II: Animal Study

Adult Sprague Dawley male (n = 12, 280–300 g, ~8 weeks of age) and female (n = 24, 230–250 g, ~8 weeks of age) rats were randomly divided into a control and a CUMS group. CUMS was applied according to our previous study (Lu et al., 2015). Briefly: this involved a three-week daily exposure to alternating stressors along with occasional overnight stressors. The following stressors were given in random order: damp bedding (300 ml of water spilled in the bedding), 40°-cage-tilt along the vertical axis, paired housing, exposure to an empty water bottle for 1 h immediately following a period of acute water deprivation (25 h from 0900 h until 1000 h the next day), stroboscopic illumination (300 flashes/min), and white noise. Body weight was assessed weekly during the CUMS procedure, and an open field test for anxiety-behavior (Chen et al., 2009) and sucrose preference test for depression-like

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