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Hypocretin/orexin loss changes the hypothalamic immune response

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ABSTRACT

Hypocretin, also known as orexin, maintains the vigilance state and regulates various physiological processes, such as arousal, sleep, food intake, energy expenditure, and reward. Previously, we found that when wild-type mice and hypocretin/ataxin-3 littermates (which are depleted of hypothalamic hypocretin-expressing neurons postnatally) were administered lipopolysaccharide (LPS), the two genotypes exhibited significant differences in their sleep/wake cycle, including differences in the degree of increase in sleep periods and in recovery from sickness behaviour. In the present study, we examined changes in the hypothalamic vigilance system and in the hypothalamic expression of inflammatory factors in response to LPS in hypocretin/ataxin-3 mice. Peripheral immune challenge with LPS affected the hypothalamic immune response and vigilance states. This response was altered by the loss of hypocretin. Hypocretin expression was inhibited after LPS injection in both hypocretin/ataxin-3 mice and their wild-type littermates, but expression was completely abolished only in hypocretin/ataxin-3 mice. Increases in the number of histidine decarboxylase (HDC)-positive cells and in *Hdc* mRNA expression were found in hypocretin/ataxin-3 mice, and this increase was suppressed by LPS. Hypocretin loss did not impact the change in expression of hypothalamic inflammatory factors in response to LPS, except for interferon gamma and colony stimulating factor 3. The number of c-Fos-positive/HDC-positive cells in hypocretin/ataxin-3 mice administered LPS injections was elevated, even during the rest period, in all areas, suggesting that there is an increase in the activity of histaminergic neurons in hypocretin/ataxin-3 mice following LPS injection. Taken together, our results suggest a novel role for hypocretin in the hypothalamic response to peripheral immune challenge. Our findings contribute to the understanding of the pathophysiology of narcolepsy.

1. Introduction

The neuropeptides hypocretin-1 and 2 (also known as orexin-A and B) are produced by hypothalamic neurons. These two neuropeptides are derived from the same precursor, prepro-hypocretin, and bind to G-protein-coupled receptors, hypocretin receptor 1 and 2. Hypocretins are involved in the regulation of various physiological processes, such as sleep/wakefulness, food intake, energy expenditure, and reward (de Lecea and Sutcliffe, 2005; Sakurai, 2007). Exclusive localization of prepro-hypocretin expression to the perifornical area of the lateral hypothalamus has been reported (Moriguchi et al., 2002; Sakurai et al., 1999).

Hypocretin signalling has been identified as one of the neural mediators against sickness behaviour. Administration of the bacterial endotoxin LPS suppresses normal hypocretin signalling and causes inactivity (Gaykema and Goehler, 2009; Grossberg et al., 2011). In our previous study, we showed that LPS-induced sickness behaviour varied depending on whether or not hypocretin neurons were present (Tanaka et al., 2015) by studying hypocretin/ataxin-3 mice in which hypocretin neurons are postnatally ablated (Hara et al., 2001). Our results suggested that sickness behaviour induced

Abbreviations: Aoc1, amine oxidase, copper containing 1; B2m, beta-2 microglobulin; Cox2, cyclooxygenase-2; Csf3, colony stimulating factor 3; Cxcl15, chemokine (C-X-C motif) ligand 15; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hcrt, hypocretin; Hdc, histidine decarboxylase; Hnmt, histamine N-methyltransferase; Ifng, interferon gamma; Il10, interleukin 10; Il18, interleukin 18; Il1b, interleukin 1 beta; Il1rn, interleukin 1 receptor antagonist; Il6, interleukin 6; Il8, interleukin 8; LPS, lipopolysaccharide; MHC, major histocompatibility complex class; Myd88, myeloid differentiation primary response 88; Nptx2, neuronal pentraxin-2; Orx, orexin; Pdyn, prodynorphin; Pmch, pro-melanin concentrating hormone; Ptges, prostaglandin E synthase; Ptgs2, prostaglandin-endoperoxide synthase 2; Tlr4, toll-like receptor 4; Tnf, tumor necrosis factor; Tnfa, tumor necrosis factor alpha.

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by peripheral immune challenge is regulated by hypocretin neurons in the hypothalamus. LPS induced a further increase in non-rapid-eye-movement sleep periods and faster recovery from sickness behaviour in these transgenic mice (Tanaka et al., 2015), suggesting that the hypothalamic immune response is affected by the loss of hypocretin neurons, which also impacts the vigilance state (Tanaka et al., 2015).

Histaminergic neurons in the hypothalamic tuberomammillary nucleus play a critical role in the maintenance of a high state of vigilance during wakefulness in concert with hypocretin neurons (Shan et al., 2015; Thakkar, 2011). Histamine is synthesized by HDC from the amino acid histidine and can be metabolized by two enzymes, HNMT and ACO1 (Schwelberger, 2004; Schwelberger, 2007). Because histamine is not a specific substrate of AOC1, it is not routinely considered in narcolepsy study in comparison with the two other enzymes. An increase in histaminergic neurons in the postmortem brains of narcoleptics has been reported (John et al., 2013). Although humans and animals with narcolepsy have similar symptoms, they differ in the changes in the number of histaminergic neurons (John et al., 2013; Valko et al., 2013). Therefore, in the present study, we examined histamine synthesis and metabolism in our animal model of inflammation to provide insight into the pathophysiology of narcolepsy.

Peripheral immunological challenge can activate central cytokine networks. Acute immunological stress induced by LPS rapidly affects hypothalamic cytokine expression (Breder et al., 1994; Castanon et al., 2004; Laye et al., 1994; Pitossi et al., 1997; Quan et al., 1998; Roche et al., 2006; Wong et al., 1997). Proinflammatory cytokines in the hypothalamus, including IL1β, IL6 and TNF, are elevated after neuroinflammation induced by LPS (Gatti and Bartfai, 1993; Laye et al., 1994; Pitossi et al., 1997). The proinflammatory cytokine IL-18 has recently emerged as a key player in neuroinflammatory and neurodegenerative processes, with wide behavioural and cognitive effects (Alboni et al., 2010; Bossu et al., 2010; Felderhoff-Mueser et al., 2005). Proinflammatory cytokines acting in the brain elicit sickness behaviours (Dantzer, 2001; Kelley et al., 2003). Furthermore, Ptgs2/Cox2 mRNA expression was found in rat brains after peripheral injection of LPS (Quan et al., 1998). COX2 functions as an inflammatory mediator to catalyse the conversion of free essential fatty acids to prostanoids, which are known vasodilators. Vasodilation via prostanoids induces the migration of lymphocytes. As a result of increased migration of lymphocytes, inflammatory diseases are exacerbated. TLR4 is the receptor for lipopolysaccharide (LPS) (Poltorak et al., 1998). Ptgs2/Cox-2 and Ptges are involved in pro-inflammatory responses downstream of TLR4/MyD88-mediated signalling.

A better understanding of the hypothalamic immune response in hypocretin/ataxin-3 transgenic mice (Hara et al., 2001) would provide insight into the pathophysiology of narcolepsy, particularly because narcoleptic patients have abnormal cytokine profiles in their periphery and cerebrospinal fluid (Dauvilliers et al., 2014; Kornum et al., 2015; Lecendreux et al., 2015; Maurovich-Horvat et al., 2014; Tanaka et al., 2014). In this study, we examined the interaction between the vigilance system and the LPS-induced hypothalamic inflammatory response using hypocretin/ataxin-3 mice (Hara et al., 2001).

2. Materials and methods

2.1. Ethics statement

All experiments were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the ethics committee on animal experiments of Kansai Medical University and Tokyo Metropolitan Institute of Medical Science.

2.2. Animals

The presence of the ataxin-3 transgene was identified by PCR genotyping using tail DNA. The expression of the ataxin-3 transgene regulated by the human prepro-hypocretin promoter selectively kills over 90% of hypocretin neurons in the hypothalamus by approximately twelve weeks of age (Hara et al., 2001; Kantor et al., 2009). Male mice (12 weeks old) were housed under a 12-h light/dark cycle with lights on from 8:00 A.M. to 8:00 P.M., corresponding to zeitgeber time (ZT) 0–12, at 22–24 °C, and were provided *ad libitum* access to food and water.

2.3. LPS injections

Twelve-week-old mice received intraperitoneal injections of LPS (Sigma-Aldrich) (250 μg/kg) dissolved in 0.9% saline or 0.9% saline alone at ZT8. The next day, at ZT1, the animals were decapitated for RT-qPCR (five wild-type littermates that received saline, five wild-type littermates that received LPS, four hypocretin/ataxin-3 transgenic mice that received saline, and four hypocretin/ataxin-3 transgenic mice that received LPS) or perfused transcardially with 0.1 M phosphate buffer (pH 7.4) followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for immunofluorescence (four animals in each group) the next day at ZT1 (17 h after LPS injections), and their brains were rapidly removed.

2.4. RT-qPCR

The hypothalamic region was dissected coronally from the optic chiasma to the mammillary bodies (−3.5 mm from the Bregma) using a brain slicer (Zivic Instruments). The dorsal limit of the hypothalamus was the roof of the third ventricle, and the lateral limit was the amygdala (Tanaka et al., 2010; Terao et al., 2006). Total RNA was isolated from each individual murine hypothalamus using Trizol reagent (Invitrogen). Single-stranded cDNA was synthesized using a PrimeScript RT reagent Kit with gDNA Eraser (Takara). The expression levels of each mRNA were determined by RT-qPCR with the Rotor-Gene Q system (Qiagen) using Thunderbird SYBR qPCR Mix (Toyobo) and gene-specific primer pairs (Table 1). The relative quantity of target gene expression was evaluated according to the ΔΔCt method with *Gapdh* as an internal control. Relative gene expression levels were compared using Student's *t*-test. A value of $P < 0.05$ was considered significant.

2.5. Immunofluorescence

Free-floating serial coronal sections, 25 μm thick, of the brain were made on a sliding freezing microtome and stored in PBS with 0.3% Triton X-100 and 0.02% sodium azide in a refrigerator until the sections were processed for immunohistochemistry. Then, the sections were incubated with rabbit anti-HDC antibody (1:1500; Progene) and goat anti-c-Fos antibody (1:100; Santa Cruz Biotechnology) diluted in PBS with 0.3% Triton X-100 overnight at room temperature. Thereafter, the sections were incubated with a donkey Alexa488-labelled anti-rabbit IgG (1:5000) and a donkey Alexa594-labelled anti-goat IgG (1:5000) (Molecular Probes). The histaminergic neurons were visualized under a fluorescence microscope equipped with a digital camera (Eclipse E1000M, Nikon) and counted.