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ALTERATIONS OF OREXINERGIC AND MELANIN-CONCENTRATING HORMONE NEURONS IN EXPERIMENTAL SLEEPING SICKNESS

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Abstract—Human African trypanosomiasis or sleeping sickness is a severe, neglected tropical disease caused by the extracellular parasite *Trypanosoma brucei*. The disease, which leads to chronic neuroinflammation, is characterized by sleep and wake disturbances, documented also in rodent models. In rats and mice infected with *Trypanosoma brucei brucei*, we here tested the hypothesis that the disease could target neurons of the lateral hypothalamus (LH) containing orexin (OX)-A or melanin-concentrating hormone (MCH), implicated in sleep/wake regulation. In the cerebrospinal fluid of infected rats, the OX-A level was significantly decreased early after parasite neuroinvasion, and returned to the control level at an advanced disease stage. The number of immunohistochemically characterized OX-A and MCH neurons decreased significantly in infected rats during disease progression and in infected mice at an advanced disease stage. A marked reduction of the complexity of dendritic arborizations of OX-A neurons was documented in infected mice. The evaluation of NeuN-immunoreactive neurons did not reveal significant neuronal loss in the LH of infected mice, thus suggesting a potential selective vulnerability of OX-A and MCH neurons. Immunophenotyping and quantitative analysis showed in infected mice marked activation of microglial cells surrounding OX-A neurons. Day/night oscillation of *c*-Fos baseline expression was used as marker of OX-A neuron activity in mice. In control animals Fos was expressed in a higher proportion of OX-A neurons in the night (activity) phase than in the day (rest) phase. Interestingly, in infected mice the diurnal spontaneous Fos oscillation was reversed, with a proportion of OX-A/Fos neurons significantly higher at daytime than at nighttime. Altogether the findings reveal a progressive decrease of OX-A and MCH neurons and dysregulation of OX-A neuron diurnal activity in rodent models of sleeping sickness. The data point to the involvement of these peptidergic neurons in the pathogenesis of sleep/wake alterations in the disease and to their vulnerability to inflammatory signaling.

Key words: orexin/hypocretin, African trypanosomiasis, Fos, sleep, wakefulness, *Trypanosoma brucei*.

INTRODUCTION

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease, still endemic in foci of sub-Saharan Africa (Simarro et al., 2010, 2012). The disease is caused by the extracellular flagellate protozoan *Trypanosoma brucei* (*T. b.*) *gambiense* (in West and Central Africa) and *T. b. rhodesiense* (in East Africa), inoculated by the bite of the tsetse fly (Kennedy, 2013; Lejon et al., 2013; Buguet et al., 2014). When T cells and the parasites cross the blood–brain barrier by an active multistep process and invade the central nervous system (CNS), the disease evolves from a first, systemic (hemolymphatic) stage to meningoencephalitis (Kristensson et al., 2010; Masocha et al., 2014; Bentivoglio and Kristensson, 2014). In the CNS, the infection, which is almost invariably fatal if left untreated, causes a chronic neuroinflammatory pathology without marked neurodegeneration (Kristensson et al., 2010).

The clinical picture of HAT includes severe neurological and psychiatric signs and symptoms, with a characteristic disruption of the sleep–wake cycle leading to diurnal somnolence and nocturnal insomnia, as well as alterations of the sleep structure (Buguet et al., 2001, 2005, 2014). Such disturbances have also been described in rats infected with *T. b. brucei* (Grassi-Zucconi et al., 1995; Darsaud et al., 2004; Seke-Etet et al., 2012), a non human-pathogenic subspecies of the parasite which is widely used, for obvious safety reasons, in rodent models of the disease.

Wakefulness and sleep states (rapid eye movement, REM, slow wave sleep, SWS) are complex functions regulated by a distributed network of neurons which reside in the brainstem, diencephalon and basal forebrain, and utilize a variety of neurotransmitters

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Abbreviations: AOI, area of interest; CSF, cerebrospinal fluid; Dpi, day post-infection; HAT, Human African trypanosomiasis; iNOS, inducible nitric oxide synthase; LD, light/dark; LH, lateral hypothalamus; LPS, lipopolysaccharide; MCH, melanin-concentrating hormone; OX, orexin; REM, rapid eye movement; RIA, radioimmunoassay; SOREM, sleep-onset REM sleep; SWS, slow wave sleep.

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(Jones, 2005; Brown et al., 2012). A key center of the arousal system is represented by the cell population of the lateral hypothalamus (LH) which contains orexin (OX)-A and B, also called hypocretin-1 and 2 (Sutcliffe and de Lecea, 2002; Sakurai, 2007; Ohno and Sakurai, 2008). These neuropeptides, cleaved from the common precursor prepro-orexin, are largely co-localized in the same neurons (Nixon and Smale, 2007), and OX-A is more stable than OX-B. Orexinergic innervation is widely distributed in the CNS, and the orexinergic system has been implicated in a variety of functions, especially the regulation of feeding and energy homeostasis, as well as sleep–wake state transitions and arousal stability (Sakurai, 2007; Ohno and Sakurai, 2008; Sakurai and Mieda, 2011; Kukkonen, 2013; Jones and Hassani, 2013; de Lecea and Huerta, 2014).

Deficiency in OX signaling, due to neuronal death or mutations in genes encoding OX receptors, causes in humans and animals the lifelong neurological disorder narcolepsy (Kilduff and Peyron, 2000; Thannickal et al., 2000; Peyron et al., 2000; Sutcliffe and de Lecea, 2002), in which immune-mediated mechanisms have been hypothesized though not yet demonstrated (Bentivoglio and Kristensson, 2007; Overeem et al., 2008; Fontana et al., 2010; Partinen et al., 2014). A low or undetectable amount of OX-A in the cerebrospinal fluid (CSF) is considered a hallmark of narcolepsy with cataplexy (sudden loss of skeletal muscle tone), reflecting dramatic loss of OX-A neurons (Mignot et al., 2002; Bourgin et al., 2008). Main disturbances in narcolepsy are represented by bouts of overwhelming daytime sleepiness, sleep fragmentation, as well as sleep-onset REM sleep (SOREM) episodes, which consist of a dysregulation of the SWS–REM sleep sequence (Nishino and Mignot, 1997; Scammell, 2003). These features were also reported in HAT patients and *T. b. brucei*-infected rats (Grassi-Zucconi et al., 1995; Darsaud et al., 2004; Seke-Etet et al., 2012). In patients affected by HAT *gambiense*, OX-A levels in the CSF have been reported to be significantly higher than in narcoleptic patients but lower than in cases with other neurological diseases, suggesting that OX-A dysfunction could be involved in HAT (Dauvilliers et al., 2008).

Peptidergic neurons which contain melanin-concentrating hormone (MCH) are intermingled with orexinergic neurons in the LH, and give rise to widespread projections reaching also sleep- and wake-regulatory cell groups (Broberger et al., 1998; Bittencourt, 2011). The MCH neuron population has been implicated in feeding behavior, locomotor activity, autonomic nervous functions, and sleep regulation (Guyon et al., 2009; Peyron et al., 2009; Tsunematsu et al., 2014). In particular, MCH neurons exert an inhibitory influence on OX-A neurons, fine-tuning their final output (Adamantidis et al., 2008; Rao et al., 2008; Burt et al., 2011), and can counteract the wake-promoting activity of arousal neurons (Konadhode et al., 2013), promoting sleep (Jones and Hassani, 2013).

Hypothalamic peptidergic neurons implicated in sleep–wake regulation were not previously investigated in *T. b.*-infected brains. On this basis, the present study was designed to test the hypothesis that African trypanosome infection could target OX-A and MCH neurons. We here measured OX-A level in the CSF of infected rats during disease progression, and we investigated OX-A and MCH neurons in the brain of the same rats and of infected mice in the advanced stage of disease. Immunophenotyping was used to analyze the activation of microglial cells in the LH of infected mice. In addition, *c*-Fos was used as functional marker of OX-A neuronal activity in infected mice. This strategy was adopted on the basis of data indicating that Fos baseline expression in OX-A neurons undergoes in rats a variation related to sleep and wakefulness states (Estabrooke et al., 2001; España et al., 2003), and is under pronounced circadian control also in mice (Marston et al., 2008).

EXPERIMENTAL PROCEDURES

Animals and infection

Young adult (3 month-old) male Sprague–Dawley rats and C57BL/6J mice were housed for at least 3 weeks before the experiment, under a 12-h/12-h light/dark (LD) cycle and controlled temperature and humidity, with free access to food and water. The animals were then randomly divided in two groups, and one group was infected by ip injections with pleomorphic *T. b. brucei* AnTat 1/1 (derived from stabilate EATRO 1125, Laboratory of Serology, Institute of Tropical Medicine Prince Leopold Antwerp, Belgium). Blood samples were obtained from the tip of the tail of all the animals to verify parasitemia in the third day post-infection (dpi).

The experiments were carried out under veterinarian assistance, minimizing the number of animals used and avoiding their suffering, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and received approval by the Animal Care and Use Committee of the University of Verona and authorization by the Italian Ministry of Health.

Experimental design

The investigation was pursued at different time points during the progression of the meningoencephalitic stage of African trypanosomiasis. In our infection paradigm *T. b. brucei*-infected rats survive 35–45 days, *T. b. brucei*-infected mice survive about 35 days, and parasite invasion of the CNS starts in both species during the second week of disease, around dpi 11–12 (Masocha et al., 2004, 2008).

Analysis of OX-A concentration in the CSF and peptidergic neurons in the LH was pursued in infected rats. Under deep anesthesia (pentobarbital, 50 mg/kg, i.p.), the CSF was harvested and the rats were then perfused, as indicated below, at dpi 18, 19, 20, 21, 22, 23, and 40. The CSF was also harvested from control, non-infected rats, matched with the infected ones at dpi 18, 23 and 40. The rats were sacrificed during daytime,