Neuroscience 188 (2011) 55–67

c-FOS EXPRESSION IN NEURONS PROJECTING FROM THE PREOPTIC AND LATERAL HYPOTHALAMIC AREAS TO THE VENTROLATERAL PERIAQUEDUCTAL GRAY IN RELATION TO SLEEP STATES

K.-C. HSIEH,[a,b] I. GVILIA,[a,c] S. KUMAR,[a,b] A. USCHAKOV,[a,b] D. MCGINTY,[a,d] M. N. ALAM[a,d] AND R. SZYMUSIAK[a,b]*

[a]*Research Service, V.A. Greater Los Angeles, CA 91343, USA*

[b]*Departments of Medicine and Neurobiology, UCLA School of Medicine, Los Angeles, CA, USA*

[c]*Beritashvili Institute of Physiology, Tbilisi, GA, USA*

[d]*Department of Psychology, UCLA, Los Angeles, CA, USA*

Abstract—The ventrolateral division of the periaqueductal gray (vlPAG) and the adjacent deep mesencephalic reticular nucleus have been implicated in the control of sleep. The preoptic hypothalamus, which contains populations of sleep-active neurons, is an important source of afferents to the vlPAG. The perifornical lateral hypothalamus (LH) contains populations of wake-active neurons and also projects strongly to the vlPAG. We examined nonREM and REM sleep-dependent expression of c-Fos protein in preoptic-vlPAG and LH-vlPAG projection neurons identified by retrograde labeling with Fluorogold (FG). Separate groups of rats (n=5) were subjected to 3 h total sleep deprivation (TSD) followed by 1 h recovery sleep (RS), or to 3 h of selective REM sleep deprivation (RSD) followed by RS. A third group of rats (n=5) was subjected to TSD without opportunity for RS (awake group). In the median preoptic nucleus (MnPN), the percentage of FG+ neurons that were also Fos+ was higher in TSD-RS animals compared to both RSD-RS rats and awake rats. There were significant correlations between time spent in deep nonREM sleep during the 1 h prior to sacrifice across groups and the percentage of double-labeled cells in MnPN and ventrolateral preoptic area (VLPO). There were no significant correlations between percentage of double-labeled neurons and time spent in REM sleep for any of the preoptic nuclei examined. In the LH, percentage of double-labeled neurons was highest in awake rats, intermediate in TSD-RS rats and lowest in the RSD-RS group. These results suggest that neurons projecting from MnPN and VLPO to the vlPAG are activated during nonREM sleep and support the hypothesis that preoptic neurons provide inhibitory input to vlPAG during sleep. Suppression of excitatory input to the vlPAG from the LH during sleep may have a permissive effect on REM sleep generation. Published by Elsevier Ltd on behalf of IBRO.

Key words: median preoptic nucleus, ventrolateral preoptic area, perifornical lateral hypothalamus, sleep deprivation.

The preoptic hypothalamus contains populations of sleep-active neurons and is considered an important sleep regulatory site (Szymusiak and McGinty, 2008). The highest densities of sleep-active neurons within the preoptic area are found in the ventrolateral preoptic area (VLPO) and median preoptic nucleus (MnPN). The MnPN and VLPO innervate the ventrolateral subdivision of the periaqueductal gray (vlPAG) (Lu et al., 2006; Uschakov et al., 2006; Yoshida et al., 2005; Saper and Levisohn, 1983), a region implicated in multiple autonomic and behavioral functions, including sleep. The medial preoptic nucleus (MPO) has strong reciprocal connections with the vlPAG (Rizvi et al., 1992, 1996; Murphy et al., 1999), as does the bed nucleus of stria terminalis (BST) (Holstege et al., 1985; Gray and Magnuson, 1992; Dong and Swanson, 2006). These projections have been implicated in autonomic and neuroendocrine functions. Projection from the preoptic hypothalamus to the vlPAG may regulate sleep-related changes in physiological variables, such as respiration and heart rate. A subset of the PAG neurons has been shown to exhibit state-dependent activity related to cardiovascular or respiratory cycles (Ni et al., 1990a,b).

The vlPAG and the area immediately ventral and lateral to it, the deep mesencephalic reticular nucleus (DpMe), have been proposed to play a pivotal role in REM sleep control (Lu et al., 2006; Luppi et al., 2006). Muscimol injections in vlPAG augment REM sleep (Sastre et al., 1996, 2000; Boissard et al., 2002; Lu et al., 2006; Vanini et al., 2007). Lesions of vlPAG and the adjacent DpMe increase REM sleep (Lu et al., 2006; Kaur et al., 2009). These findings suggest that the vlPAG/DpMe plays an inhibitory role in gating the onset and/or maintenance of REM sleep. Kaur et al. (2009) have proposed that sleep-active neurons in preoptic hypothalamus provide inhibition of the vlPAG, allowing REM sleep generating neurons in the pontine reticular formation to become activated.

To investigate patterns of activation in projection neurons from the preoptic area to vlPAG in relation to sleep states, we combined retrograde tracer injection into the vlPAG with c-Fos protein immunohistochemistry in the

*Correspondence to: R. Szymusiak, Research Service (151A3), V.A. Greater Los Angeles Healthcare System, 16111 Plummer Street, North Hills, CA 91343, USA. Tel: +1-818-891-7711 ext 7568; fax: +1-818-885-9575.
E-mail address: rszym@ucla.edu (R. Szymusiak).

Abbreviations: AW, active waking; BST, bed nucleus of stria terminalis; DpMe, deep mesencephalic reticular nucleus; FG, Fluorogold; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; MnPN, median preoptic nucleus; MPO, medial preoptic nucleus; QW, quiet waking; rMnPN, rostral part of the median preoptic nucleus; RS, recovery sleep; RSD, REM sleep deprivation; SWD, spike and wave discharge; TSD, total sleep deprivation; vlPAG, ventrolateral division of the periaqueductal gray; VLPO, ventrolateral preoptic area; VLPOc, core VLPO.

0306-4522/11 $ - see front matter. Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2011.05.016

preoptic area in rats. We examined two conditions designed to cause differential expression of deep nonREM versus REM sleep; 3 h total sleep deprivation (TSD) followed by 1 h of recovery sleep and 3 h selective REM sleep deprivation (RSD) followed by 1 h of recovery sleep. These conditions were compared with groups of rats subjected to TSD without opportunity for recovery sleep.

In contrast to the preoptic area, neuronal activity in the lateral hypothalamus (LH) is strongly wake-related (Alam et al., 2002; Scammell et al., 2000; Mileykovskiy et al., 2005; Lee et al., 2005). Hypocretin (orexin) neurons in the LH project to the vlPAG (Peyron et al., 1998) and are implicated in the regulation of arousal and suppression of REM sleep (see Sakurai et al., 2010; Bonnavion and de Lecea, 2010 for review). We therefore examined c-Fos expression as an indicator of neuronal activity in vlPAG projection neurons located in the perifornical LH across the three experimental groups.

EXPERIMENTAL PROCEDURES

All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. All protocols were reviewed and approved by the Animal Care and Use Committee at the Veterans Administration of the Greater Los Angeles Health Care System. Subjects were male Sprague–Dawley rats weighing between 300–350 g at the time of surgery.

Animal surgery

All surgical procedures were performed under deep isoflurane anesthesia. Retrograde tracer, Fluorogold (FG; Fluorochrome, Denver, CO, USA), was pressure-injected into the vlPAG stereotaxically (bregma AP −7.4, ML −0.7, DV −6.4 mm) with a Hamilton microsyringe controlled by an infusion pump (KDS310, KD Scientific Inc, Holliston, MA, USA). 10 nl of FG solution (4% in dH_2O) was infused at a rate of 0.01 μl/min, and the injection needle was slowly withdrawn after waiting additional 10 min at the end of infusion. EEG and EMG electrodes were implanted at the time of tracer injection. Small bone screws placed through the skull in contact with the dura at frontal and parietal areas were used to record EEG. EMG was recorded with insulated stainless steel wires placed in the muscles of the dorsal neck. All leads were connected to a plastic connector that was cemented to the skull. Animals were allowed to recover for 1 week before being subjected to polygraph recording.

Sleep recording and analysis

A recording cable connected the implanted headplug to a 12-pin commutator (SL12C, Plastics One, Roanoke, VA, USA). EEG and EMG signals were conducted by fully grounded cables, amplified with differential AC amplifiers (Model 1700, A-M Systems, Sequim, WA, USA), bandpassed in 1–30 Hz and 10–1000 Hz respectively, and digitized with a CED 1401 Plus interface unit (Cambridge Electronic Design, Cambridge, England) sampled in 256 Hz before storing into computer hard drive. The polygraphic recording was visually scored with a Spike2 script (Sleepscore v2.02 by Dr. Geoff Horseman) offline.

Sleep-wake behaviors were scored in one of the six stages (AW, QW, N1, N2, Tran, REM) in 10 s epochs. Active waking (AW) was defined by presence of spontaneous behaviors such as grooming or locomotion, elevated and variable EMG activity in the presence of a desynchronized EEG, while quiet waking (QW) was defined by desynchronized EEG and diminished EMG tonic activity, which typically displayed as an intermediate stage between AW and sleep. Light nonREM sleep (N1) was scored when moderate slow wave activity was present in the EEG, and deep nonREM sleep (N2) was scored when high amplitude slow wave activity occupied more than half of the epoch duration. The transitional stage between NREM and REM (Tran) was defined by high amplitude theta waves intermixed with reduced amplitude slow wave activity in the EEG and low levels of EMG tone. Since Tran almost always precedes the beginning of a fully expressed REM sleep episode, scoring Tran has been shown to improve the analysis of sleep structure (Benington et al., 1994). REM sleep was defined by presence of desynchronized EEG, presence of theta waves in EEG and muscle atonia (minimal EMG activity). Wake represents combined AW and QW, and NREM represents combined N1, N2 and Tran. The EEG power spectrum analysis for delta (1.0–4.0 Hz) was carried out using a Spike2 script provided by CED (SUDSA ver2.2) for each 10 s epoch of artifact-free EEG during deprivation and recovery periods (Fig. 1).

Procedures

Animals were housed individually in Plexiglass recording chambers (9 in.×9 in.×10 in.) that were placed inside larger incubators (internal dimension: 23 in.×18 in.×49 in.). Ambient temperature in the incubator was 23±1 °C and a 12/12 light/dark cycle was maintained. Animals were adapted to the recording environment for 9–11 days and to the recording cable for at least 3 days before the experiment. Food and water were continuously available during all adaptation and experimental procedures.

Three experimental groups were used in this study. Separate groups of rats were subjected to either 3-h TSD (n=5) or 3-h selective RSD (n=5) starting at the onset of light period (ZT0), followed by 1-h undisturbed recovery sleep (RS) prior to sacrifice (TSD-RS and RSD-RS, respectively). A third group of rats were subjected to 2-h TSD without opportunity for recovery sleep prior to the sacrifice (n=5). Two rats in this awake group were sacrificed at ZT4, the same circadian time as TSD-RS and RSD-RS rats. The other three rats in the awake group were sacrificed during the dark phase at ZT16. As single- and double-labeled cell counts were found to be similar in awake rats sacrificed at ZT4 and ZT16, data from the five awake rats were combined for statistical comparison with the TSD-RS and RSD-RS groups.

TSD and RSD were achieved by tapping the Plexiglass recording cage using a stick placed through a port in the incubator that housed the recording cage. This was done whenever animals showed signs of entering either nonREM or REM sleep in the continuous monitoring by polygraph and video camera. The timing of each cage tap was logged by a keystroke at computer keyboard and subjected to analysis as a measure of accumulating sleep pressure (see Fig. 1). At the end of recovery period, the incubator was opened and the animal was promptly anesthetized with i.p. pentobarbital injection (100 mg/kg; Nembutal, Lundbeck Inc., Deerfield, IL, USA).

Histology and immunohistochemical procedures

Under deep anesthesia of sodium pentobarbital, rats were perfused transcardially with 100 ml of 0.1 M phosphate buffered isotonic saline (PBS), followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1 M PB (pH=7.4). The brains were then immersed in same fixative for additional 1 h before being stored in the 30% sucrose in 0.1 M PB in 4 °C for at least 2 days. The brains were then rapidly frozen with dry ice and cut in 30-μm coronal sections with a Leica SM2400 microtome. The sections were rinsed and then immersed in cryoprotectant, and stored at −20 °C until staining.

Fos protein and FG immunostaining were carried out in sequence on free-floating sections using an ABC method with Nickel-DAB and DAB as chromogens, respectively. According to the information provided by the manufacturer, the anti-c-Fos antibody (PC-38 rabbit polyclonal IgG, Calbiochem, San Diego, CA, USA) was raised against a synthetic peptide corresponding to amino