Title: Role of polysialylated neural cell adhesion molecule in rapid eye movement sleep regulation in rats.

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ABSTRACT

Recent evidence suggests that synaptic plasticity occurs during homeostatic processes including sleep-wake regulation, although underlying mechanisms are not well understood. Polysialylated neural cell adhesion molecule (PSA NCAM) is a transmembrane protein that has been implicated in various forms of plasticity. To investigate whether PSA NCAM is involved in the neuronal plasticity associated with spontaneous sleep-wake regulation and sleep homeostasis, three studies were conducted using rats. First, we showed that PSA NCAM immunoreactivity is present in close proximity to key neurons in several nuclei of the sleep-wake system, including the tuberomammillary hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus. Second, using Western blot analysis and densitometric image analysis of immunoreactivity, we found that 6 h of sleep deprivation changed neither the levels nor the general location of PSA NCAM in the sleep-wake system. Finally, we injected endoneuraminidase (endo N) intracerebroventricularly to examine the effects of PSA removal on sleep-wake states and EEG slow waves at both baseline and during recovery from 6 h of sleep deprivation. Endo N-treated rats showed a small but significant decrease in baseline rapid eye movement (REM) sleep selectively in the late light phase, and a facilitated REM sleep rebound after sleep deprivation, compared to saline-injected controls. Non-REM sleep and wake were unaffected by endo N. These results suggest that PSA NCAM is not particularly involved in the regulation of wake or non-REM sleep, but plays a role in the diurnal pattern of REM sleep as well as in some aspects of REM sleep homeostasis.

INTRODUCTION

Although synaptic plasticity has been shown to be an integral component of the cellular mechanisms underpinning learning and memory, it is also involved in the regulation of homeostatic functions, such as energy balance and sleep (Pinto et al., 2004; Horvath & Gao, 2005; Citri & Melanka, 2008). For example, in orexin (also known as hypocretin)-containing neurons, which have a pivotal role in promoting wake and stabilizing behavioural states (Sakurai, 2007), 4 h of sleep deprivation (SD) increases spontaneous excitatory postsynaptic currents and causes long term potentiation-like changes in evoked synaptic currents (Rao et al., 2007). The action of noradrenaline on orexinergic neurons changes from excitatory to inhibitory after only 2 h of SD (Grivel et al., 2005; see also Modirrousta et al., 2005). In the basal forebrain, which plays a role in cortical and behavioural activation and sleep homeostasis (Kaur et al., 2008; Kalinchuk et al., 2008), 3 h of SD upregulates $GABA_A$ receptors on cholinergic neurons (Modirrousta et al., 2007), and 6 h of SD alters levels of proteins involved in synaptic function and cellular structure (Basheer et al., 2005). These findings suggest that synaptic plasticity, including rapid synaptic rewiring and remodeling, occurs during acute SD and perhaps even during normal sleep-wake regulation.

The polysialylated form of neural cell adhesion molecule (PSA NCAM) has been implicated in various forms of neural plasticity (Rutishauser, 2008; Bonfanti & Theodosis, 2009). Abundant in the late embryonic/early neonatal brain, PSA NCAM is largely downregulated in the adult brain but continues to be present at high levels in certain areas where it has been shown to play a crucial role in certain types of plasticity (Bonfanti, 2006; Burgess et al., 2008). For example, hippocampal long-term potentiation (Becker et al., 1996; Muller et al., 2000) and structural remodeling in certain hypothalamic nuclei following estradiol treatment (Parducz et al., 1993; Hoyk et al., 2001) and during lactation and dehydration (Theodosis et al., 1994, 1999) are abolished following the enzymatic removal of PSA from NCAM by endoneuraminidase (endo N). Further, PSA NCAM levels have been shown to increase in an activity-dependent manner in several brain regions after stimulation, e.g., in the hippocampus and prefrontal cortex after learning (Lopez-Fernandez et al., 2007; Ter Horst et al., 2008), and in the suprachiasmatic nucleus (SCN), the site of the principal circadian clock in mammals, after brief photic stimulation (Glass et al., 2003) or glutamate administration (Prosser et al., 2003).

Given the role of PSA NCAM in neural plasticity and the evidence for synaptic plasticity associated with sleep regulation, we asked whether PSA NCAM has a role in sleep-wake regulation in adult rats. We used immunohistochemistry to investigate the presence of PSA NCAM in the sleep-wake system. To determine whether short-term SD alters PSA NCAM levels, we used Western blot analysis of the hypothalamus and densitometric analysis of immunoreactivity in select brain regions following 6 h of SD. Finally, polygraphic recordings were used to determine whether the enzymatic removal of PSA NCAM from the entire brain alters spontaneous sleep-wake and/or recovery sleep following 6 h of SD.

METHODS

Animals

Adult male Wistar rats (Charles River Canada, St. Constant, QC, Canada), 275-350 g (Experiments 1 and 4) or 350-400 g (Experiments 2 and 3) at the time of surgery, were housed in

pairs in standard polypropylene cages at the animal care facility, under a 12:12 light:dark cycle (lights on at 07.00 h) at 23 ± 1ºC. Food and water were available ad libitum throughout the experiments. All animal handling procedures were conducted in keeping with the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Dalhousie University Committee on Laboratory Animals.

Experiment 1 – PSA NCAM immunoreactivity in sleep-wake nuclei of the adult rat brain

Immunohistochemistry

The specificity of the antibodies used is described in Supplementary Table 1.

Following one week of acclimation to the animal care facility, animals (n = 2) were given an overdose of an anaesthetic mixture (ketamine, 120 mg/kg; xylazine, 6.4 mg/kg; acepromazine, 1.2 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.2-7.4). The brains were post-fixed for 3 h in the same fixative, cryoprotected overnight in 30% sucrose in phosphate-buffered saline, and then cut into five sets of 40 µm sections from the genu of the corpus callosum to the locus coeruleus on a freezing microtome.

For single PSA NCAM labeling, sections were incubated overnight at room temperature in a mouse monoclonal antibody raised against PSA NCAM (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After 3 rinses with Tris-buffered saline, the sections were incubated for 70 min in a Cy3-tagged donkey anti-mouse IgM secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA, USA), rinsed again, and then mounted on gelatin-coated slides. The slides were air-dried and coverslipped (Cytoseal 60; Richard-Allan Scientific, Kalamazoo, MI).

For double-labeling, selected sections were labeled first as above for PSA NCAM, and then for additional neurotransmitter markers of key **sleep/wake-promoting neurons** including: choline acetyltransferase (1:50; Chemicon, Temecula, CA, USA) to label cholinergic neurons in the basal forebrain; orexin B (1:500; Phoenix, Burlingame, CA, USA) to label orexinergic neurons **and melanin-concentrating hormone (MCH; 1:1600; Phoenix, Burlingame, CA, USA) to label MCH-containing neurons in the perifornical region of the lateral hypothalamus**; adenosine deaminase (1:200; Chemicon) to label histaminergic neurons in the tuberomammillary nucleus; serotonin transporter (1:5000; Chemicon) to label serotonergic neurons in the dorsal raphe nucleus; and tyrosine hydroxylase (1:6000; Pel-Freez, Rogers, AR, USA) to label noradrenergic neurons in the locus coeruleus. These transmitter markers were visualized by using the same protocol as for PSA NCAM but with appropriate Cy2-conjugated IgG secondary antibodies. Non-specific secondary antibody staining was assessed by omitting the primary antibodies, and was absent for all the secondary antibodies used.

Fluorescence microscopy

Sections single-labeled for PSA NCAM were examined with a Zeiss Axiovert 200 microscope equipped with an Axiocam Hrc camera (Zeiss). Sections double-labeled for PSA NCAM and the neurotransmitter markers were examined with a Zeiss 510 laser scanning confocal microscope. Images were acquired with a 63x oil objective lens. An argon laser and a HeNe1 laser were used to capture Cy2 (excitation and emission filters at 488 nm and 505-550 nm, respectively) and Cy3 (excitation and emission filters at 543 nm and 560-615 nm, respectively) signals, respectively. The immunostaining in the 40 µm sections was examined using 1 µm Z-stack optical sections.

Experiment 2 – PSA NCAM protein levels in the hypothalamus following SD

SD protocol

For 5 days prior to the day of SD, rats (n = 16) were brought up by 08.00 h from the animal care facility and transferred in pairs to the experimental cages for acclimation to the experimental set-up. The rats remained undisturbed inside the cages for 6 h, and were then returned to the animal care facility. The cages were made of plexiglass (37.5 x 37.5 x 37.5 cm^3), and placed inside wooden boxes (57.5 x 45.0 x 47.5 cm^3) equipped with a fan, a light, and a window (which allowed for the observation of the rats) on the front. The lighting cycle was the same as in the animal care facility.

On the day of SD, rats were transferred in pairs to the experimental cages. One half of the rats (n = 8) was kept awake by gentle handling for 6 h, starting at one h after lights on (08.00 h). The rats were kept awake by tapping on the cage, moving the bedding around inside the cage, and introducing novel objects into the cage; rats were not directly touched during SD. The rats were disturbed only when they showed behavioural signs of sleep (i.e., relaxed position with closed eyelids). The other half of the rats (n = 8) were left undisturbed for 6 h, thus serving as controls; the behaviours of these rats were scored every 10 min as either wake or sleep, and it was confirmed that they were mostly asleep. A cohort of two sleep deprived and two control rats was studied in parallel on each day of experiment.

Western blot and densitometry

Immediately following SD, control and sleep deprived animals were anaesthetized with isoflurane and decapitated. Brains were weighed and dissected on an ice-cooled plate. The hypothalamus was removed, weighed, frozen in liquid nitrogen, and then stored at -80°C until processing. For Western immunoblotting, the tissue was thawed and homogenized in Hepes buffer (10 mM, pH 7.9) containing $MgCl_2$ (1.5 mM), KCl (10 mM), dithiothreitol (0.5 mM), and aprotinin (a protease inhibitor; 10 µg/ml), and centrifuged at 10 000 x g for 20 min at 4°C. All chemicals were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada) except for aprotinin (Roche Pharmaceuticals, Indianapolis, IN, USA). The resulting supernatant was considered to be the cytosolic fraction. To isolate the membranous fraction, pellets were re-suspended by vortex and sonication in RIPA buffer that contained, in addition to the Hepes buffer, $NaPO_4$ (10 mM), 2.0% EDTA, 1.0% Triton X-100, 0.1% sodium dodecyl sulphate, and 0.5% sodium deoxycholate. The preparation was centrifuged at 20 000 x g for 30 min at 4°C. Membranous fraction supernatants were assayed for protein content using a Bio-Rad Protein Assay Kit (Pierce; Rockford, IL, USA).

To perform gel electrophoresis, the membranous fractions were first mixed with loading buffer containing 12% sodium dodecyl sulphate and 6% mercaptoethanol. Two different protocols were then used: 1) the samples were heated to 95°C for 5 min (Crown et al., 2005); or 2) the samples were stored overnight at 4°C (Seki & Arai, 1991). Equal amounts of protein were loaded into each lane. Gel electrophoresis was used to separate the protein on an 8% resolving gel for 1.5-2 h at 100 V.

Proteins were transferred from the gel to a polyvinylidine fluoride membrane (Bio-Rad; Mississauga, ON, Canada) for 1.5 h at 365 mA. After rinsing with Tris-buffered saline, membranes were blocked for one h in 5% non-fat dry milk and then incubated overnight at 4°C in a mouse monoclonal anti-PSA NCAM primary antibody (1:1000, Chemicon; or 1:10, Developmental Studies Hybridoma Bank; Supplementary Table 1). Membranes were washed in

Tris-buffered saline, incubated for one h in donkey anti-mouse IgM antibody that was conjugated to peroxidase, and rinsed. Enhanced Chemiluminescence Plus® (Amersham Biosciences, Quebec City, QC) was applied to the membranes for 5 min, after which the blot was developed using a gel and image blot scanner (STORM®, Amersham Biosciences). Following the above procedures, blots were stripped with Blot Restore (Chemicon) and then incubated with an anti-cadherin antibody (1:2000; Abcam, Cambridge, MA, USA; Supplementary Table 1) to reveal levels of cadherin protein, which served as a loading control, using the same procedures as for PSA NCAM.

After immunoblot development, the relative optic density of PSA NCAM immunoreactivity compared to cadherin immunoreactivity was determined for each respective lane using ImageQuant TL Software (Amersham Biosciences).

Experiment 3 – PSA NCAM immunoreactivity levels in the hypothalamus following SD

The SD protocol was the same as for Experiment 2, with four sleep deprived and four control animals.

Immunofluorescence and densitometry

Immediately following SD, animals were perfused under anesthesia and brain sections were obtained as described in Experiment 1. Sections were single-labeled for PSA NCAM using a mouse monoclonal PSA NCAM antibody (1:1000; Chemicon; Supplementary Table 1). All comparable sections were processed concurrently. The images of selected sections were examined with a Zeiss Axiovert 200 microscope, coupled to an Axiocam Hrc camera (Zeiss). All images were acquired using a 10x objective, under the same lighting conditions. These images were converted to grayscale in Adobe Photoshop, and densitometric analyses were conducted to obtain integrated density values using Image J software (National Institutes of Health, Bethesda, MD, USA). The regions for analysis were selected based on preliminary analyses; all regions with PSA NCAM immunoreactivity under control and SD conditions were examined. All densitometric analyses were conducted by an experimenter blind to the treatment conditions.

Experiment 4 – Sleep and wake parameters at baseline and following 6 h of SD in endo N-treated rats

Rats were individually handled for 5 min per day for 4-5 days prior to both surgery and habituation to the experimental cages.

Endo N intracerebroventricular (icv) injection

Rats were anaesthetized by an i.p. injection of ketamine (60 mg/kg), xylazine (3.2 mg/kg), and acepromazine (0.6 mg/kg) and placed in a stereotaxic apparatus. Depth of anaesthesia was continually monitored during the surgery by visually checking for signs of increased respiration and/or withdrawal response to a pinch of a paw or the tail. The skull was exposed, a small hole was drilled into it, and a Hamilton syringe with a 26 gauge fixed needle was lowered into the right lateral ventricle [0.8 mm posterior, 1.5 mm lateral, and 3.5 mm ventral to bregma (Paxinos and Watson, 1998)]. The syringe was filled with 2.5 µl of either physiological saline (n = 5) or endo N (40 U/µl, 100 U total; n = 6). A pilot study in which we tested two different doses of endo N (100 U and 200 U; Petridis et al., 2004) showed that an icv injection of 100 U of endo N virtually abolished PSA immunoreactivity from the adult rat brain 2

weeks post-injection (n = 2). The injection was made at a rate of 0.5 µl/min; the needle was left in the injection site for 5 min after infusion to minimize spillage along the needle and was then removed slowly.

EEG/electromyogram (EMG) electrode implantation

Immediately following the injection of saline or endo N, two stainless steel EEG electrodes were screwed into the skull to be in contact with the dura above the left frontal (2.0 mm anterior and 2.0 mm lateral to bregma) and right parietal (4.0 mm posterior, and 2.0 mm lateral to bregma) cortices. A ground electrode was embedded into the skull at 2.0 mm posterior to lambda. EMG electrodes were made of insulated stainless steel wire (AS 636, Cooner Wire, Chatsworth, CA, USA) that was exposed 2-3 mm and were bilaterally implanted in the nuchal muscles with the exposed area in direct contact with the muscles. All electrodes were fitted into a miniature connector (Plastics One; Roanoke, VA, USA), which was secured to the skull with dental cement.

Polygraphic recordings

Following an eight day post-surgery recovery period, animals were individually transferred to the experimental cages (see Experiment 2). After 24 h, the animals were connected to the recording cables for acclimation. Forty-eight h later (i.e., 12 days following surgery and endo N injection), a 24 h baseline EEG and EMG recording began at 07.00 h (lights on). After the baseline recording, at 13 days post-injection, animals were sleep deprived for 6 h, starting at 07.00 h (i.e., the first half of the light phase) using the same gentle handling protocol as in Experiment 2, and were then allowed an 18 h recovery period.

EEG and EMG signals were amplified by a Grass P511 amplifier (Grass-Telefactor, West Warwisk, RI, USA), and filtered (high- and low-pass filters set at 0.3 Hz and 10 kHz, respectively, for the EEG signal, and 1 Hz and 10 kHz, respectively, for the EMG signal). All signals were converted from analogue to digital at a sampling rate of 256 Hz and digital data were stored with SleepSign software (Kissei Comtec, Irvine, CA, USA) for off-line analysis.

Sleep-wake data analysis

Polygraphic recordings were auto-scored for non-rapid eye movement (NREM) sleep, REM sleep, and wake in 10-sec epochs. An epoch was scored as NREM sleep if it consisted of >50% EEG delta waves (0.5 – 4 Hz) and low integrated EMG activity. REM sleep was scored if an epoch was composed of >30% theta waves (5 – 8 Hz) and very low integrated EMG activity. If none of these conditions was met, the epoch was scored as wake. The autoscored data was manually checked and, in case of disagreement, the visual score was considered correct.

The amount of time that each animal spent in wake, NREM and REM sleep was summed in 2-h blocks across the 48 h of recording. The mean duration and the number of NREM and REM sleep episodes were also calculated for each 2-h period over the 48 h recording.

A power spectral analysis of EEG delta waves (0.5-4 Hz) during NREM sleep, which are markers of NREM sleep intensity and drive (Borbely, 1982), was conducted using a fast Fourier transform (Hanning; 2 sec window). EEG delta power during NREM sleep was averaged in 2-h bins for the first 6 h following SD and for the corresponding baseline period (i.e., the second half of the light phase).

Immunohistochemistry

At day 14 post-injection and immediately following the recovery period after SD, rats were given an overdose of a ketamine/xylazine/acepromazine mixture and perfused, as described in Experiment 1. Brain sections from 0.8 to 8.8 mm posterior to bregma (Paxinos & Watson, 1998) were immunostained for PSA NCAM as in Experiment 1, with the exception of the use of a different mouse monoclonal anti-PSA NCAM antibody (1:1000, Seki & Arai, 1993; Supplementary Table 1). Fluorescent sections were examined microscopically as described in Experiment 1.

Statistical analyses

For Experiment 2, the relative optic densities of Western blots were compared between SD and control conditions using independent samples t-tests. For Experiment 3, the results of the densitometric analyses of PSA NCAM immunofluorescence between SD and control animals were compared using Mann-Whitney U tests.

For Experiment 4, repeated measures ANOVAs, one with within factor Phase (light and dark) and between factor Treatment, and another with within factor Time (2 h bins during the light and dark period) and between factor Treatment, were used to analyze the amount of wake, NREM sleep, and REM sleep, as well as the number and duration of NREM and REM sleep episodes at baseline. A double repeated measures ANOVA with two within factors, Condition (Baseline, Recovery) and Time (6 h intervals), was used to analyze any differences in the sleep parameters within the saline group and within the endo N group. The amount of EEG delta power during NREM sleep in the first 6 h period after SD was expressed as a percentage of values from the time-matched baseline period, and analyzed with a repeated measures ANOVA (within factors Condition and Time and between factor Treatment). Tukey post hoc tests were used to further analyse significant effects and interactions. Statistical significance was considered to be achieved if $P < 0.05$.

RESULTS

Experiment 1 - PSA NCAM immunoreactivity in the sleep-wake system

Within the wake-promoting system, PSA NCAM immunoreactivity was seen in several regions of the hypothalamus and brainstem. The PSA NCAM immunoreactivity appeared to be present either on the plasma membrane or in the extracellular space, and not in the cytoplasm of neurons within the sleep-wake system. Of the regions of the sleep-wake system that contained PSA NCAM immunoreactivity, the tuberomammillary nucleus and locus coeruleus showed the most intense immunoreactivity. Both cell bodies and dendrites of histaminergic (adenosine deaminase-immunoreactive, Fig. 1C) and noradrenergic (tyrosine hydroxylase-immunoreactive Fig. 1E) neurons were surrounded by strong PSA NCAM immunoreactivity. The PSA NCAM staining in these nuclei was not confined but extended to surrounding areas, i.e., the mammillary nuclear complex and the parabrachial nuclei, respectively. The dorsal raphe nucleus was moderately immunoreactive for PSA NCAM; the PSA NCAM labeling surrounded cell bodies of serotonergic neurons, but its association with their dendrites occurred less frequently (Fig. 1D). Some PSA NCAM immunoreactivity was also observed in the laterodorsal tegmental nucleus, which contains cholinergic neurons. In all of these areas, some non-principal neurons were also surrounded by PSA NCAM immunostaining.

The PSA NCAM immunoreactivity in the perifornical lateral hypothalamus was the least dense of all regions within the wake-promoting system that showed any significant amount of PSA NCAM immunoreactivity. Sparse, punctate labels surrounded some, but not all, of the orexin- (Fig. 1B) and MCH-containing cell bodies. Notably, PSA NCAM immunostaining was very sparse in the cholinergic regions of the basal forebrain (e.g., the medial septum, substantia innominata and the magnocellular basalis nucleus), and immunoreactivity, if present, was not associated with cholinergic neurons.

Within the brain regions involved in sleep promotion, moderate PSA NCAM immunoreactivity was found in the ventral region of the median preoptic nucleus (Fig. 1A), where immunolabeling predominantly surrounded cell bodies of neurons. PSA NCAM labeling was notably sparse in the ventrolateral preoptic nucleus. In REM sleep-regulatory areas, PSA NCAM labeling was low in the dorsal region of the ventrolateral periaqueductal gray, and virtually absent in the dorsal part of the deep mesencephalic reticular nucleus and in the sublaterodorsal nucleus.

PSA NCAM immunoreactivity was also found in various regions that are not directly involved with sleep-wake regulation, including the hippocampus, piriform cortex, amygdala, as well as the paraventricular and ventral lateral geniculate nuclei of the thalamus. Additionally, strong immunoreactivity was seen in regions of the hypothalamus such as the median eminence, the supraoptic nucleus, and the SCN throughout its rostrocaudal extent (Fig. 1F). The walls of the lateral and third ventricles were strongly immunoreactive. Very little PSA NCAM labeling was found in the neocortex. These observations corroborate previous reports (Bonfanti et al., 1992; Seki & Arai, 1993; Glass et al., 2003; Prosser et al., 2003).

Experiment 2 - Effects of 6 h of SD on levels of PSA NCAM protein in the hypothalamus

We used Western blot analyses on the protein obtained from homogenized hypothalamic samples to determine any quantitative changes in the amount of PSA NCAM following 6 h of SD (Fig. 2A). There was no significant difference in the relative optic density of PSA NCAM in the hypothalamus of sleep deprived (n = 8) versus control (n = 7) animals (one-tailed t-test: t_{13} = 1.07, P = 0.31; Fig. 2B). Similar results were obtained using two different PSA NCAM antibodies (see Supplementary Table 1) and two different Western blot protocols (heating vs. cooling the samples after placing them in buffer; see the Methods).

Experiment 3 - Effects of 6 h of SD on levels of PSA NCAM immunoreactivity in the brain

Although we did not find any changes in the PSA NCAM protein amount in the entire hypothalamus after 6 h of SD using Western blot analysis, region-specific changes in PSA NCAM amounts that are undetectable with Western blots might still be occurring. To investigate this possibility and extend the study to include the entire sleep-wake system in the brain, PSA NCAM immunoreactivity was measured, using densitometry, in various hypothalamic and other sleep-wake regulatory nuclei that showed moderate to bright immunoreactivity for PSA NCAM in both sleep deprived and control animals. The areas that were examined included: the median preoptic nucleus, optic chiasm, SCN, retrochiasmatic area, arcuate nucleus, median eminence, perifornical lateral hypothalamus, tuberomammillary nucleus (dorsal part), dorsal raphe nucleus, ventrolateral periaqueductal gray, and locus coeruleus. In addition, the PSA NCAM immunoreactivity in the hippocampus, an area that normally shows high PSA NCAM immunoreactivity, was quantified for comparison. There was no significant change in the

intensity of PSA NCAM immunofluorescence in any of these areas except the median eminence. The density of PSA NCAM immunoreactivity in the median eminence was significantly higher in animals sleep deprived for 6 h compared to control rats (Mann-Whitney U = 1.00, P = 0.04). Visual inspection of PSA NCAM immunoreactivity in the rest of the sleep-wake system and across the neuraxis indicated no obvious differences in the intensity or localization (i.e., there were no areas in which PSA was detected only before or only after SD) of PSA NCAM between the control and sleep deprived animals.

Experiment 4 – Effects of icv injection of endo N on sleep-wake regulation

In this experiment, rats received icv injections of saline or endo N to remove PSA from the entire brain and to assess its effect on sleep-wake regulation. At day 12 post-injection, a 24 h baseline EEG/EMG recording was taken, and at day 13 post-injection, animals were sleep deprived for 6 h during the first half of the light phase, and then allowed to recover for 18 h. All rats were observed periodically throughout the experiment and we did not notice any signs of abnormal behaviour; they all seemed to eat, drink, and groom normally. An analysis of the change in body weight from the time of surgery to the time of perfusion in a subset of rats (n = 4 each for saline and endo N) showed that both treatment groups gained similar amounts of weight during the 2 weeks (saline: +44 ± 22 g, endo N: +50 ± 8 g; mean ± standard deviation).

In saline-treated animals, the pattern and intensity of PSA NCAM immunoreactivity were similar to those observed in Experiment 1. In animals that received a right lateral ventricle injection of 100 U of endo N, PSA NCAM immunoreactivity was virtually eliminated in most (~90%) areas of the brain (Fig. 3). PSA NCAM immunoreactivity was reduced only by about 75% in the subventricular zone of the lateral ventricle and the median eminence of the hypothalamus.

The basic features of the EEG and EMG activities during wake, NREM sleep and REM sleep in endo N-treated animals appeared indistinguishable from those of the saline group throughout the entire 48 h recording period including baseline, SD, and recovery. Thus, scoring of behavioural states in endo N-treated animals was straightforward and we did not encounter any problems.

Baseline wake

During the baseline recording period, endo N-treated rats showed the normal diurnal rhythm of wake expected of nocturnal rodents, as did saline-control animals. There was no significant difference in wake amounts between the saline and endo N rats in either the 12 h (i.e, the light or dark phase; Fig. 4A) or 2 h (Fig. 4B) bin analysis.

Baseline NREM sleep

Similar to wake, endo N-treated rats showed a normal diurnal pattern of NREM sleep, and there were no significant differences between the two groups in the amount of time spent in NREM sleep in either the 12 h (Fig. 4C) or at any 2-h bin during the light or the dark phase (Fig. 4D). Likewise, the frequency and mean duration of NREM sleep episodes were similar in both groups (Fig. 5A-D).

Baseline REM sleep

Although a basic diurnal rhythm of REM sleep amount was visible in both the saline and endo N-treated rats, the pattern was different between the groups (Time x Treatment interaction,

$F_{5, 45} = 3.0$, P = 0.021; Fig. 4F). The amount of REM sleep in control animals continued to increase in the second half of light phase, whereas in endo N-treated rats it began to decline after 8 h from lights on, and tended to remain lower than in controls until 2 h after lights off. Post hoc testing showed that endo N-treated rats spent significantly less time in REM sleep than saline-treated rats during the last 2 h of the light phase (P = 0.016).

In parallel with the amount of REM sleep, endo N-treated animals showed a significantly lower number of REM sleep episodes, compared to saline, during the last 2 h of the light cycle (Time x Treatment interaction, $F_{5, 45} = 3.6$, P = 0.008; saline > endo N at Zeitgeber time or ZT 12, P 0.018; Fig. 5F). In addition, endo N-treated rats tended to show shorter mean durations of REM sleep episodes particularly in the light phase than in controls (P = 0.07) and, overall, less diurnal variation in the duration of REM sleep episodes than the saline-treated rats (Fig. 5 G,H).

Sleep deprivation

The SD procedures used were highly effective in preventing almost all sleep during the first half of the light phase (Fig. 7). Specifically, 6 h of SD by "gentle handling" led to a 95% decrease, compared to baseline, in NREM sleep in saline and endo N rats. REM sleep was completely suppressed in all rats.

The number of times the animals needed to be aroused was scored in 1 h bins over the 6 h of SD. Figure 6 shows that rats required an increasing number of stimulation as the SD progressed, particularly after the third hour, with no significant difference between the two groups.

Recovery wake

After SD, both saline- and endo N-treated animals spent significantly less time in wake, compared to their respective time-matched baseline values, during the first 6 h (i.e., second half of the light phase; Saline: $F_{1, 4} = 227.3$, P = 0.001; endo N: $F_{1, 5} = 34.1$, P = 0.002) and the second 6 h (i.e., first half of the dark phase; Saline: $F_{1, 4} = 23.1$, P = 0.009; endo N: $F_{1, 5} = 99.8$, P = 0.001; Fig. 7A-B') of recovery. Wake amounts were still below baseline values in the third 6 h period of recovery (i.e., second half of the dark phase) in endo N-treated animals ($F_{1, 5} = 9.8$, P = 0.026). The amount of wake lost during recovery and its time course were similar in both groups (Fig. 8A).

Recovery NREM sleep

The latency to the first NREM episode following the 6 h of SD was similar in both treatment groups (Saline: 1.8 ± 0.7 min, endo N: 5.8 ± 2.8 min; $t_9 = 1.29$, P = 0.23). Both saline- and endo N-treated rats showed significantly more NREM sleep across the first 6 h (by up to 52% in both groups; Saline: $F_{1, 4} = 202.1$, P = 0.001; endo N: $F_{1, 5} = 25.3$, P = 0.004) and the second 6 h (by up to 50-57% in both groups; Saline: $F_{1, 4} = 26.9$, P = 0.007; Endo N: $F_{1, 5} = 81.1$, P = 0.001) recovery periods, as compared to baseline values (Fig. 7C-D'). During the third 6 h of recovery, the NREM sleep amount in saline-treated rats was not significantly different from baseline (P = 0.12), but endo N-treated rats continued to spend significantly more time in NREM sleep (by 31%; $F_{1, 5} = 9.1$, P = 0.026). The NREMS gain was similar in both groups (Fig. 8B).

In both groups, the increase in the amount of NREM sleep in the first 6 h after SD was due to longer durations, and not increased frequencies, of NREM sleep episodes. In fact, in both treatment groups, the number of NREM sleep episodes during this period significantly decreased (by up to 46-48%), compared to baseline (Saline: $F_{1, 4} = 326.5$, P = 0.001; endo N: $F_{1, 5} = 91.0$, P

= 0.001; Table 1). In contrast, the mean duration of NREM sleep episodes increased by 176-200%, compared to baseline, in both treatment groups (Saline: $F_{1, 4} = 56.0$, $P = 0.002$; Endo N: $F_{1, 5} = 91.9$, $P = 0.001$; Table 1).

During the second 6 h period after SD, the number of NREM sleep episodes showed a significant increase over baseline in the saline group ($F_{1, 4} = 19.0$, $P = 0.01$; Table 1). The mean duration of NREM sleep episodes during this period showed a small but significant increase in the endo N group ($F_{1, 5} = 9.2$, $P = 0.029$). In the last 6 h of the recovery period, both the frequency and the mean duration of NREM sleep episodes returned to baseline levels in both groups.

Recovery REM sleep

As was seen with NREM sleep latency, the latency to the first REM sleep episode following the 6 h of SD was similar in both groups (Saline: 26.1 ± 7.0 min, endo N: 25.6 ± 6.1 min; $t_9 = 0.06$, $P = 0.96$). Saline-treated animals did not show any significant change in the amount of REM sleep, compared to the baseline, during the first ($P = 0.68$) and the second ($P = 0.12$) 6 h of the recovery period. A significant increase in REM sleep amount was seen only in the third 6 h recovery period in these animals (by 91%; $F_{1, 4} = 12.6$, $P = 0.024$; Fig. 7E'). In contrast, endo N-treated animals showed a REM sleep rebound over baseline during the first (by 50%; $F_{1, 5} = 79.6$, $P = 0.001$), second (by 100%; $F_{1, 5} = 24.1$, $P = 0.004$), and third (by 64%; $F_{1, 5} = 6.8$, $P = 0.047$) 6 h periods after SD (Fig. 7F,F'). The REMS gain during the first 6 h of recovery was significantly higher in endo-N rats than in saline controls (Group x Time, $F_{1,18} = 154.4$, $P = 0.019$; post hoc $P < 0.002$) , and a similar trend was observed during the second 6 h recovery period (Fig. 8C).

Saline and endo N-treated rats exhibited different temporal patterns of increases in the number and/or the duration of REM sleep episodes (Table 1). During the first 6 h of recovery, endo N-treated animals showed an increase in the mean duration of REM sleep episodes compared to baseline (by 37%; $F_{1, 5} = 10.84$, $P = 0.022$), contributing to the significant increase in REM sleep amount. In the second 6 h of recovery, the endo N-treated animals showed a significant increase in the number of REM sleep episodes (by 100%, $F_{1, 5} = 54.9$, $P = 0.001$), while in the last 6 h period, they no longer showed a significant change in either the duration or the number of REM sleep episodes, relative to baseline (Table 1). In contrast, the saline group showed an increase in the mean duration of REM sleep episodes in the second ($F_{1, 4} = 34.9$, $P = 0.004$) and third ($F_{1, 4} = 8.45$, $P = 0.04$) 6 h recovery periods.

EEG delta power in recovery NREM sleep

EEG delta power (0.5-4 Hz) during NREM sleep during the first 6 h of recovery was normalized as percentage changes from baseline values from the time-matched baseline period, in 2 h intervals. There were no differences in normalized NREM sleep delta power between the two groups during the 6 h recovery period. All rats showed a higher value of normalized EEG delta power during the first 6 h of recovery than during baseline ($F_{1, 1} = 173.6$, $P = 0.0001$). The time course of delta power over the first 6 h recovery period paralleled the decrease in NREMS amount (Fig. 7C,D), with a gradual decline in EEG delta power in both groups (Time, $F_{2, 18} = 158.4$, $P = 0.001$), and delta power was highest during the first 2 h after SD in both groups ($P = 0.001$ vs. second and third 2 h intervals).

DISCUSSION

The main original findings of this study are as follows: 1) Moderate to intense PSA NCAM immunoreactivity is present in adult rats in several key areas for sleep-wake regulation, including the NREM sleep-promoting median preoptic nucleus (ventral part) and the wake-promoting tuberomammillary nucleus, dorsal raphe nucleus, and locus coeruleus; 2) Six h of SD during the first half of the light cycle altered neither the amount of PSA NCAM protein in the hypothalamus, nor the density of PSA NCAM immunoreactivity in the hypothalamus and throughout the sleep-wake system; 3) The enzymatic removal of PSA NCAM from the brain by icv injections of endo N selectively decreased the amount of baseline REM sleep in the late light phase, at 2 weeks post-injection; 4) Following 6 h of SD, saline-injected controls did not show REM sleep rebound until the last 6 h of the 18 h recovery period, but endo N-treated animals showed an increase in REM sleep immediately and throughout the entire 18 h; 5) Endo N treatment did not affect the amount of wake, NREM sleep, or EEG delta wave activity (a measure of sleep drive and intensity) either at baseline or after SD. These results indicate that PSA NCAM is present in certain brain regions involved in sleep-wake regulation in the adult rat brain and that PSA NCAM plays a role in the diurnal and homeostatic regulation of REM sleep, but probably not in the regulation of NREM sleep or wake.

PSA NCAM immunoreactivity is present in the sleep-wake system of adult rats

This is the first demonstration of the presence of PSA NCAM in select sleep-wake regulatory nuclei of the adult rat brain. The results with the three antibodies closely corroborated each other. PSA NCAM immunoreactivity was found in one NREM sleep-promoting nucleus, namely, the median preoptic nucleus (ventral part). Neurons in this nucleus increase firing during NREM and REM sleep (Suntsova et al., 2002) and project to the wake-promoting perifornical lateral hypothalamus, dorsal raphe nucleus and locus coeruleus (Uschakov et al., 2007). Median preoptic nucleus neurons have also been shown to play a role in the homeostasis of REM sleep (Gvilia et al., 2006a,b). Further, PSA NCAM immunoreactivity was observed in several, but not all, wake-promoting nuclei examined. Intense staining was found in the dorsal and ventral tuberomammillary nucleus and the locus coeruleus, whereas moderate staining was found in the dorsal raphe nucleus. Less staining was found in the perifornical lateral hypothalamus and the ventrolateral periaqueductal gray, which has recently been characterized as containing REM-off neurons (Lu *et al.*, 2006; Luppi *et al.*, 2006; Sapin *et al.*, 2009). Very little immunoreactivity for PSA NCAM was detected in the cholinergic regions of the basal forebrain, which is known for its role in sleep homeostasis (Kaur et al., 2008; Kalinchuk et al., 2008) and in the dorsal part of the deep mesencephalic reticular nucleus and the sublaterodorsal nucleus, both of which have been implicated in the regulation of REM sleep (Boissard *et al.*, 2003; Lu *et al.*, 2006; Luppi *et al.*, 2006; Sapin *et al.*, 2009).

Polysialylation of NCAM reduces cell-to-cell adhesion and facilitates neural plasticity (Rutishauser & Landmesser, 1996; Johnson et al., 2005). The presence of PSA NCAM immunoreactivity in certain sleep-wake regulatory nuclei, therefore, suggests that PSA NCAM might be fulfilling a neuroplasticity-related role in those areas. For example, PSA NCAM may allow for the synaptic remodeling that might be involved in state-dependent synaptic responses observed in orexinergic neurons (see Introduction), and perhaps in the switch between NREM sleep and REM sleep (Saper et al., 2005; Lu et al., 2006). We observed close association of PSA NCAM with histaminergic, noradrenergic, and serotonergic neurons, but the exact location of

PSA NCAM relative to the cell membranes of these neurons needs to be investigated in order to understand the specific role of PSA NCAM in the function of these wake-promoting neurons.

SD does not alter PSA NCAM levels in the hypothalamus and the sleep/wake system

PSA NCAM levels have been shown to increase after stimulation in several brain regions in an activity-dependent manner (see Introduction). We thus initially hypothesized that a 6 h SD protocol, known to induce various forms of neural plasticity (see Introduction) as well as sleep rebound, would alter the amount of PSA NCAM in the hypothalamus and other sleep/wake-promoting areas. However, the only brain region examined that showed a difference (an increase) after SD was the median eminence. The median eminence is an important neuroendocrine "gate" between the hypothalamus and the pituitary gland (Swanson, 2002), but its role in sleep-wake regulation is unclear. It is conceivable that the increase in PSA NCAM in this nucleus was due to stress that is inherent in any SD procedure, and not a result of sleep loss per se. However, it is not known whether acute stress affects PSA NCAM levels in the median eminence although chronic, but not acute, restraint stress results in an increase in PSA NCAM levels in the piriform cortex (Nacher et al., 2004) and hippocampus in rats (Sandi et al., 2001; Pham et al., 2003). The significance of the increase in PSA NCAM in the median eminence after SD remains unclear.

One possible explanation for the lack of change in the level of PSA NCAM protein or immunoreactivity in any sleep-wake area examined is that 6 h of SD was not sufficient. However, 6 h of SD by gentle handling was sufficient to cause a large sleep rebound in the present study, and it has been shown to increase the levels of proteins, such as SNAP-25, that have a role in synaptogenesis and synaptic remodeling in the rat basal forebrain (Basheer et al., 2005). Further, phosphorylated GAP-43 was up-regulated after 6 h of SD, suggesting that post-translational modifications of proteins can occur within 6 h of SD (Basheer et al., 2005). Others showed that a 30 min light pulse during the dark phase caused a significant upregulation of PSA NCAM protein in the hamster SCN within 1 h (Glass et al., 2003). These findings suggest that the impact of 6 h SD on cellular and molecular functions can be observed within a relatively short period of time, and we interpret the present results as indicating that the molecular mechanisms that mediate the impact of 6 h SD do not involve changes in the amount of PSA NCAM. However, we cannot exclude the possibility that the subcellular distribution of PSA NCAM is reorganized as a result of SD, i.e., down-regulation of PSA NCAM at one synaptic site and up-regulation of PSA NCAM at another synaptic site, which is consistent with synaptic remodeling. Such a redistribution would not necessarily lead to a change in the overall amount of the molecule (Dalva et al., 2007), nor would it be detectable by the means employed in these experiments. Electron microscopic analysis would be required for adequate study of such reorganization.

Enzymatic removal of PSA NCAM alters the diurnal pattern of baseline REM sleep

To determine more directly whether PSA NCAM is involved in sleep-wake regulation, endo N was intracerebroventricularly injected to remove PSA from NCAM, and sleep-wake states were recorded and analyzed during a 24 h baseline and during an 18 h recovery period following 6 h of SD. Immunohistochemical staining confirmed that PSA was largely (~90%) absent from the brain at 14 days post-injection (i.e., at the end of 18 h recovery period). This result is consistent with previous reports indicating that endo N at similar doses effectively

removed PSA NCAM for up to 18 days after microinjection into the SCN in adult rats (Glass et al., 2000) and up to 3 weeks after icv injection in newborn mice (Seki & Rutishauser, 1998).

In the present study, icv injections of endo N did not affect baseline amounts of wake or NREM sleep, but decreased the amount of baseline REM sleep at a specific time of the light phase. Specifically, endo N-treated animals failed to show a normal increase in REM sleep amount towards the end of the light phase and instead showed a premature decline in REM sleep. This decrease in REM sleep was due mainly to a decrease in the number, not the mean duration, of REM sleep episodes. Thus, endo N-treated animals showed less attempts to enter REM sleep in the late light phase. One explanation for this effect is that icv injections of endo N impaired the circadian signal that normally influences the timing of REM sleep occurrence.

An increase in REM sleep towards the end of the light phase has been postulated to be controlled mainly by the circadian process (McCarley & Massaquoi, 1986; Dijk & Czeisler, 1995; Wurts & Edgar, 2000). This increase in REM sleep propensity is due to an increase in the number, rather than the duration, of REM sleep episodes (Wurts & Edgar, 2000; present study). SCN lesions are known to abolish this circadian rhythm of REM sleep propensity (Wurts & Edgar, 2000). Interestingly, a study using knockout mice reported that genetic deletions of NCAM and PSA disrupted locomotor rhythms under constant (free-running) conditions (Shen et al., 2001), suggesting that PSA NCAM plays a role in stabilizing circadian rhythms of locomotor activity, and likely, sleep-wake states. The SCN has high levels of PSA NCAM immunoreactivity, particularly during the light phase (Prosser et al., 2003; Glass et al., 2003), and icv injections of endo N effectively remove PSA from the SCN (present study), which could affect circadian signaling. A recent study showed that the SCN undergoes rhythmic ultrastructural remodeling under a light:dark 12:12 condition in rats (Becquet et al., 2008). This remodeling involves the retraction and extension of glial processes that regulate the apposition of SCN neuronal perikarya with axon terminals. Currently there is no evidence that PSA NCAM is required for this remodeling; however, this glial remodeling is similar to that observed in other PSA NCAM-rich nuclei with homeostatic and neuroendocrine functions, and PSA NCAM is required for the glial remodeling in those nuclei (see Introduction). These findings suggest a role of PSA NCAM in the SCN in remodeling process, and this remodeling might play a key role for the stable expression of circadian rhythmicity in the SCN's output signal.

It is not clear how the effect of endo N on baseline sleep is specific to REM sleep. One possibility is that the circadian influences on REM and NREM sleep are anatomically distinct, and differentially susceptible to perturbation. This possibility is supported by several lines of evidence. Anatomical tracing studies have shown that (indirect) output pathways from the SCN to the parasympathetic vs. sympathetic systems originate from segregated cell populations in the SCN; these segregated pathways may become active at different times of day to produce adaptive circadian influence on the periphery (Kalsbeek et al., 2007). Furthermore, the SCN is thought to promote both wake and sleep, but at different times of day or night (Mistlberger, 2005). Although SCN neurons are known to be generally more active in the light phase, SCN neurons also show behavioural state-dependent activity patterns, increasing firing during wake and REM sleep compared to NREM sleep (Mistlberger et al., 2002; Deboer et al., 2003). A large body of literature indicates that SCN lesions do not alter the amounts of wake or sleep in rats (Mistlberger, 2005), but one study reported that SCN lesions increased NREM sleep while decreasing REM sleep (Mendelson et al., 2003). Collectively, these findings suggest that SCN neurons that promote REM sleep may be separate from those promoting NREM sleep or wake, and each mechanism might be differentially vulnerable to insult, such as removal of PSA.

Enzymatic removal of PSA NCAM may selectively facilitate recovery REM sleep following SD

In the present study all animals, regardless of treatment, showed an immediate and prolonged increase in the amount of NREM sleep during the 18 h recovery period after 6 h of SD. All rats also showed a strong increase in EEG delta power during recovery NREM sleep, particularly during the first 2 h after SD, and an increase in the mean duration of NREM sleep episodes, indicating an increase in the intensity and consolidation of post-SD sleep. These results are consistent with those obtained from intact rats (Tobler & Borbely, 1990; Gvilia et al., 2006b; Huber et al., 2007; Vyazovskiy et al., 2007). The number of interventions required to keep animals awake during SD was also similar between the treatment groups, and there was no difference in the latency to enter the first NREM and REM sleep episodes following SD. Although a more detailed analysis of the time course of sleep need build-up during SD will be useful, these results collectively suggest that the build up of sleep need during SD and the homeostatic increase in NREM sleep and EEG delta power during recovery were unaffected by endo N treatment.

The major difference in the pattern of rebound sleep between the two groups was seen in REM sleep. Whereas saline-injected controls did not show a REM sleep rebound until the third 6 h recovery period following the cessation of SD, endo N-treated animals showed a prompt REM sleep recovery (within the first 6 h) and maintained high amounts of REM sleep at least for 18 h (total recording period) after SD. Although REM sleep is known to be controlled more strongly by the circadian, rather than the homeostatic, process, it is unlikely that this REM sleep rebound in endo N animals is related to any possible impairment of REM sleep circadian regulation. This is because REM sleep rebound in endo N animals was visible during the first 6 h recovery period, i.e., during the second half of light phase, when the circadian promotion of REM sleep is normally the highest. Therefore, a lack of circadian drive at this time in endo N-treated rats (see above) would disfacilitate, rather than facilitate, REM sleep rebound.

It is possible that any facilitation of REM sleep recovery in the endo N-treated rats may be related to their lower (albeit non-significant) amount of total REM sleep during baseline, compared to the saline-treated rats (due to less REM sleep towards the end of the light phase), as detected at 12 days post-injection. This is a relatively small deficit (~10% less than in controls over 24 h) and it is unknown whether endo N-treated animals continuously had such smaller amounts of REM sleep since endo N injection. However, if that was the case, this cumulative REM sleep deficit could cause a greater REM sleep-specific homeostatic response to total SD. This possibility of cumulative REM sleep deficit can be investigated by examining the time course of sleep-wake patterns after icv injections of endo N.

Another explanation for the REM sleep response is that the removal of PSA in the dorsal raphe nucleus or the locus coeruleus led to the lack of synaptic plasticity which, through unknown mechanisms, facilitates recovery REM sleep selectively. The brainstem serotonergic and noradrenergic neurons have long been thought to play a permissive role in REM sleep as REM-off neurons that inhibit REM-on neurons during wake and NREM sleep (McCarley, 2007). It is conceivable that diminished neural plasticity (e.g., lack of changes in input configuration and failure to sustain an optimal level of excitability) in these monoaminergic nuclei following endo N treatment renders these neurons less capable of exerting inhibition. The median preoptic nucleus, which contains relatively high amounts of PSA NCAM, has been shown to play a role in REM sleep homeostasis, possibly via reciprocal connections with these monoaminergic brainstem neurons (see above). It is possible that the removal of PSA in this nucleus facilitates

recovery REM sleep. These possibilities can be investigated by injecting endo N directly into each of these nuclei and observing rebound REM sleep after SD.

Conclusions

This study demonstrates for the first time that PSA NCAM is present in select key nuclei of the sleep-wake regulatory system. Although neither the level nor the general location of PSA NCAM appears to change following 6 h of SD, the removal of PSA from the brain alters the diurnal pattern of REM sleep, suggesting an impairment of the circadian influence on REM sleep. The removal of PSA also selectively facilitated REM sleep recovery after 6 h of SD. These findings suggest a role of PSA NCAM in the diurnal pattern of REM sleep and in REM sleep homeostasis.

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AUTHORS' CONTRIBUTIONS

M.A.B. conducted the experiments and prepared the manuscript. M.A.B., S.D., and K.S. designed the experiments and interpreted the data. S.D. and K.S. edited the manuscript. K.S. conceived the original study. T.S. supplied mAb12E3 anti-PSA NCAM antibody and technical advice; D.R.M. provided materials, equipment use, and technical advice; U.R. supplied endo N and related technical advice; V.F.R. provided materials, general and technical advice. All authors read the final draft of the manuscript.

LIST OF ABBREVIATIONS

EEG	electroencephalogram
EMG	electromyogram
endo N	endoneuraminidase
icv	Intracerebroventricular
ip	intraperitoneal
NREM	non-rapid eye movement
PSA NCAM	polysialylated neural cell adhesion molecule
REM	rapid eye movement
SCN	suprachiasmatic nucleus
SD	sleep deprivation
ZT	Zeitgeber time

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Table 1: Number and duration of NREM and REM sleep episodes during baseline and recovery. The mean (± SEM) number and duration of NREM and REM sleep episodes during baseline and recovery at Zeitgeber time 7-12 (second half of the light phase), 13-18 (first half of dark phase), and 19-24 (second half of the dark phase). The duration regards a single episode. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to respective baseline.

Figure legends

Figure 1: PSA NCAM immunoreactivity is present in sleep- and wake-promoting nuclei and the suprachiasmatic nucleus in the adult rat brain. A fluorescent micrograph image shows moderate PSA NCAM immunostaining is found in the ventral region of the median preoptic nucleus (A). B,C: Images of single 1 μm optical sections shows that PSA NCAM immunoreactivity in the perifornical lateral hypothalamus (B) is sparse, but some punctate labeling is visible around orexinergic neurons (arrow, inset) and that intense PSA NCAM immunoreactivity is present in the dorsal tuberomammillary nucleus (C), surrounding both adenosine deaminase-positive (histaminergic, long arrow) and unidentified neurons (asterisk). D,E: Projection images (10 x 1 μm) of adjacent sections shows that PSA NCAM surrounds serotonin transporter-positive (serotonergic) neurons in the dorsal raphe nucleus (D, arrows) and the cell bodies of tyrosine hydroxylase-positive (noradrenergic, arrow) neurons in the locus coeruleus (E). A fluorescent micrograph image shows strong PSA NCAM immunoreactivity is seen in the suprachiasmatic nucleus (F). 3V = third ventricle; MnPO = median preoptic nucleus; oc = optic chiasm; SCN = suprachiasmatic nucleus. Scale bars = 100 μm (A,D); 20 μm (B,C,E); 200 μm (F).

Figure 2: There is no change in the amount of PSA NCAM protein in the hypothalamus following 6 h of sleep deprivation. (A) A representative Western blot is shown for hypothalamic PSA NCAM levels in two control (C) and two sleep-deprived rats (SD). The amount of PSA NCAM was normalized to the value for cadherin in the same lane. (B) Densitometric analyses of Western blots show that there is no significant difference in the relative optic density of PSA NCAM in the hypothalamus between controls (white, n = 7) and sleep-deprived rats (black, n = 8, $P > 0.05$).

Figure 3: An icv injection of endo N virtually abolishes PSA NCAM immunoreactivity in the entire brain. In a rat injected with an icv injection of saline (left), strong PSA NCAM immunostaining is seen in the median preoptic nucleus (A), the dorsal tuberomammillary nucleus and an adjacent area facing the third ventricle (B), and the locus coeruleus (C). In a rat injected with an icv injection of endo N (right), virtually all PSA NCAM immunoreactivity is abolished in the same areas (A'-C'). 3V = third ventricle; 4V = fourth ventricle; ac = anterior commissure; DTMN = dorsal tuberomammillary nucleus; LC, locus coeruleus; MnPO = median preoptic nucleus. Scale bars = 100 μm (A,C); 50 μm (B).

Figure 4: Amount (mean ± SEM) of wake, NREM sleep, and REM sleep during the 24 h baseline recording, as presented for 12 h light and 12 h dark phases (left) and in 2 h intervals (right). Saline (white, n = 5) and endo N-treated rats (black, n = 6) spent the same amount of time in wake (A,B) and NREM sleep (C,D) across the baseline recording period. However, Endo

N-treated animals showed less REM sleep during the last 2 h of the light cycle (F), compared to the saline-treated animals. The background shading indicates the dark phase. * $P < 0.05$ relative to saline.

Figure 5: Number and duration (mean ± SEM) of NREM and REM sleep episodes at baseline. Saline- and endo N-treated rats did not differ in the number or the mean duration of NREM sleep episodes during any 12 h (A,C) or 2 h interval (B,D) at baseline. However, endo N-treated rats had less REM sleep episodes than controls during the last 2 h of the baseline light phase (F). The two groups did not differ in the mean duration of REM sleep episodes (G), although endo N-treated animals tended to show shorter durations than the controls during the light phase and less diurnal variation (H). The background shading indicates the dark period. (*$P < 0.05$)

Figure 6: There is no difference between saline- and endo N-treated groups in the number of interventions required during SD to keep animals awake. Regardless of treatment, the mean (±SEM) number of interventions increased as the SD progressed, particularly in the second half.

Figure 7: Amount of wake, NREM sleep, and REM sleep during the recovery period after 6 h of SD in saline- (left) and endo N-treated rats (right). The mean (±SEM) number of minutes spent in each vigilance state is shown: top, amount in 2 h intervals across the 24 h baseline period (white circles), during 6 h of sleep deprivation (SD), and during the 18 h recovery period (black circles); bottom, amount in 6 h intervals during the 18 h recovery period (black bars) and corresponding baseline period (white bars). (A,A') Saline-treated rats showed a significant decrease in wake amount during the first 12 h of recovery, whereas Endo N-treated rats (B,B') were awake significantly less for the entire 18 h of recovery. (C,C') Saline-treated animals showed more NREM sleep during the first 12 h of recovery, whereas endo N-treated animals (D,D') showed more NREM sleep during the full 18 h of recovery. (E,E') Saline-treated rats did not show a significant REM sleep rebound until the last 6 h of recovery, whereas endo N-treated animals (F,F') showed REM sleep rebound during the entire 18 h recovery period. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to respective baseline).

Figure 8: The change (in min, mean ± SEM) in the amount of wake, NREM sleep, and REM sleep during recovery relative to baseline in 6 h intervals. The amount of each vigilance state during the recovery period was subtracted by the amount during the corresponding baseline period. While both saline (white) and endo N-treated rats (black) showed a similar loss in wake (A) and a similar gain in NREM sleep (B) amount during recovery, endo N-treated rats showed a larger gain in REM sleep amount (C), compared to saline-injected animals, during the first 6 h recovery and, to some extent, during the second 6 h recovery. (* $P < 0.0001$).

Figure 1

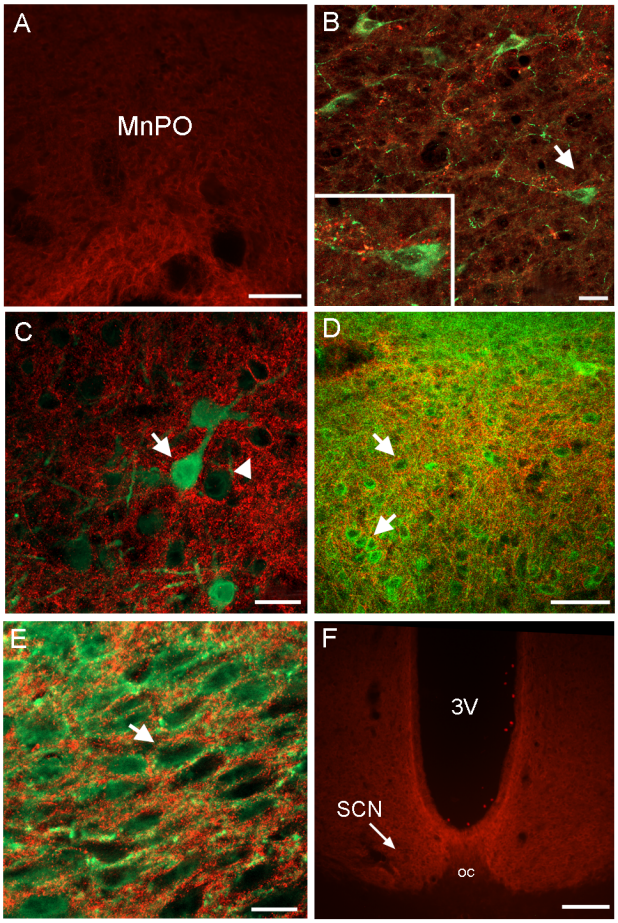
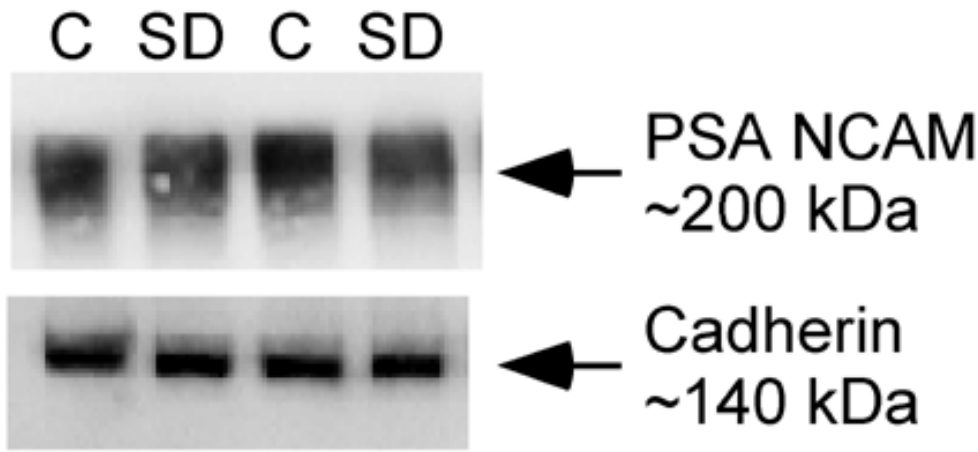


Figure 2

A


C SD C SD
PSA NCAM
~200 kDa
Cadherin
~140 kDa

B

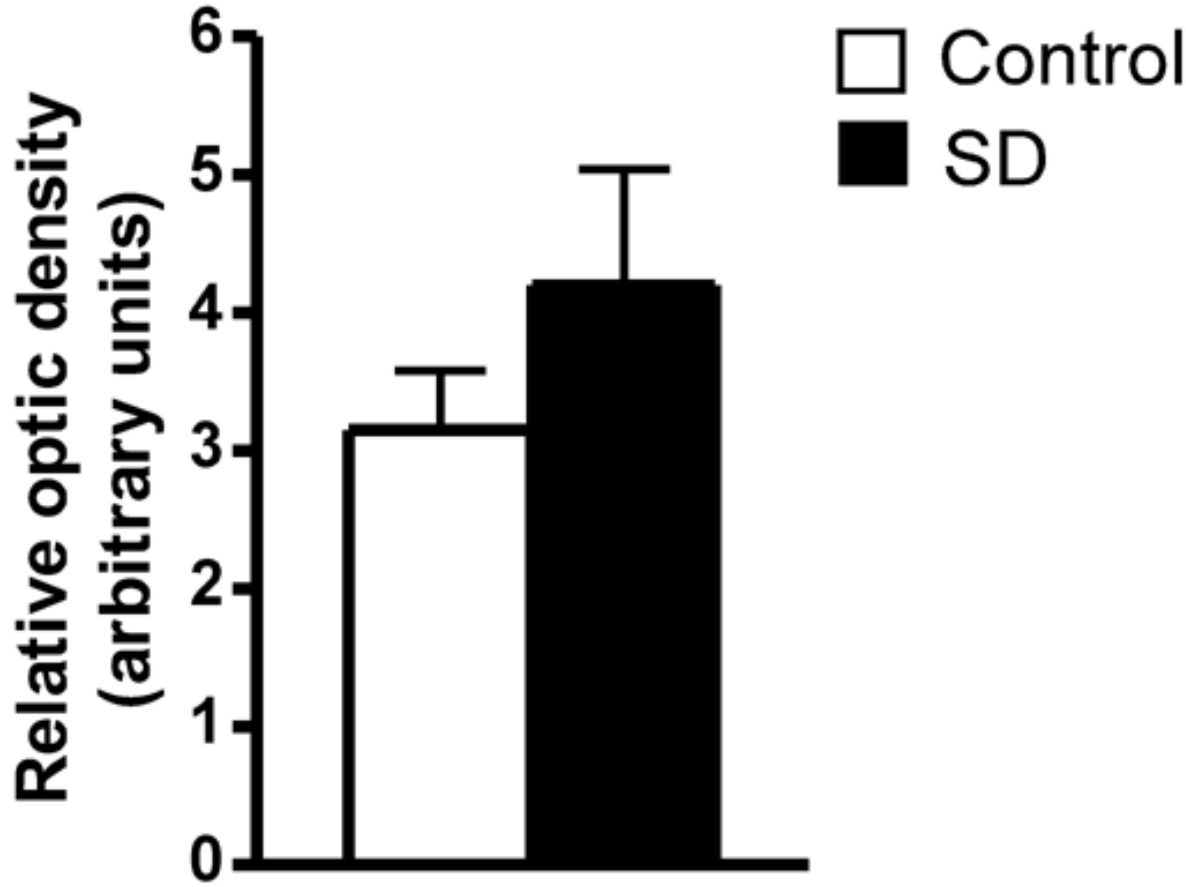

Relative optic density
(arbitrary units)
0
1
2
3
4
5
6
Control
SD

Figure 3

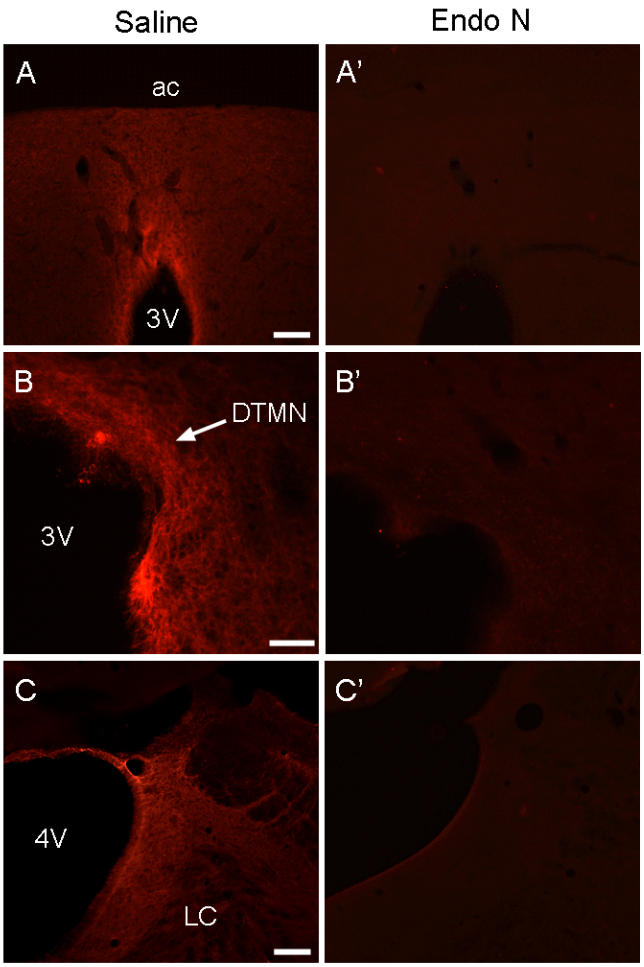


Figure 4

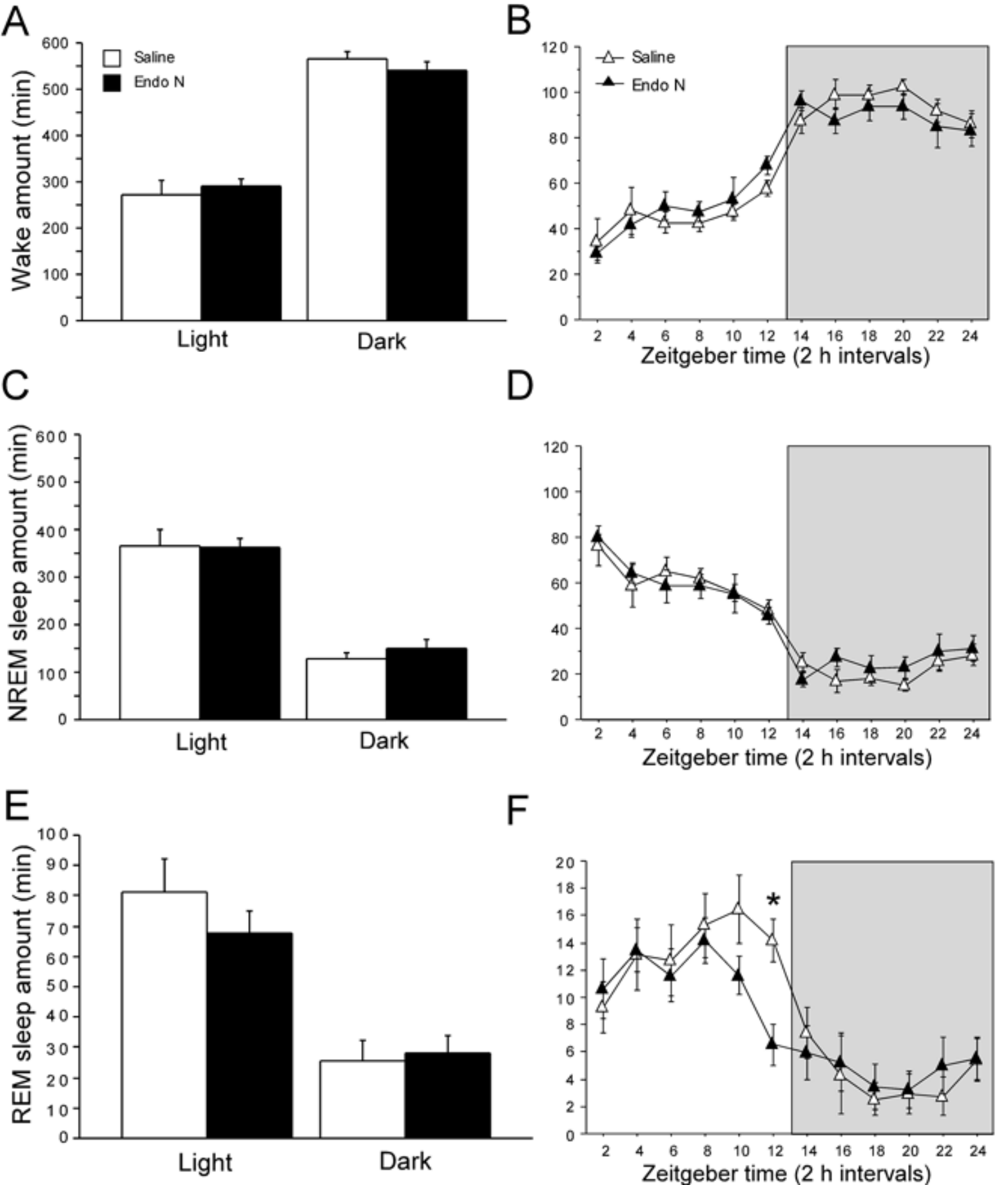

A
Saline
Endo N
Wake amount (min)
600
500
400
300
200
100
0
Light
Dark
B
Saline
Endo N
120
100
80
60
40
20
0
2 4 6 8 10 12 14 16 18 20 22 24
Zeitgeber time (2 h intervals)
C
NREM sleep amount (min)
600
500
400
300
200
100
0
Light
Dark
D
120
100
80
60
40
20
0
2 4 6 8 10 12 14 16 18 20 22 24
Zeitgeber time (2 h intervals)
E
REM sleep amount (min)
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
F
*
20
18
16
14
12
10
8
6
4
2
0
2 4 6 8 10 12 14 16 18 20 22 24
Zeitgeber time (2 h intervals)

Figure 5

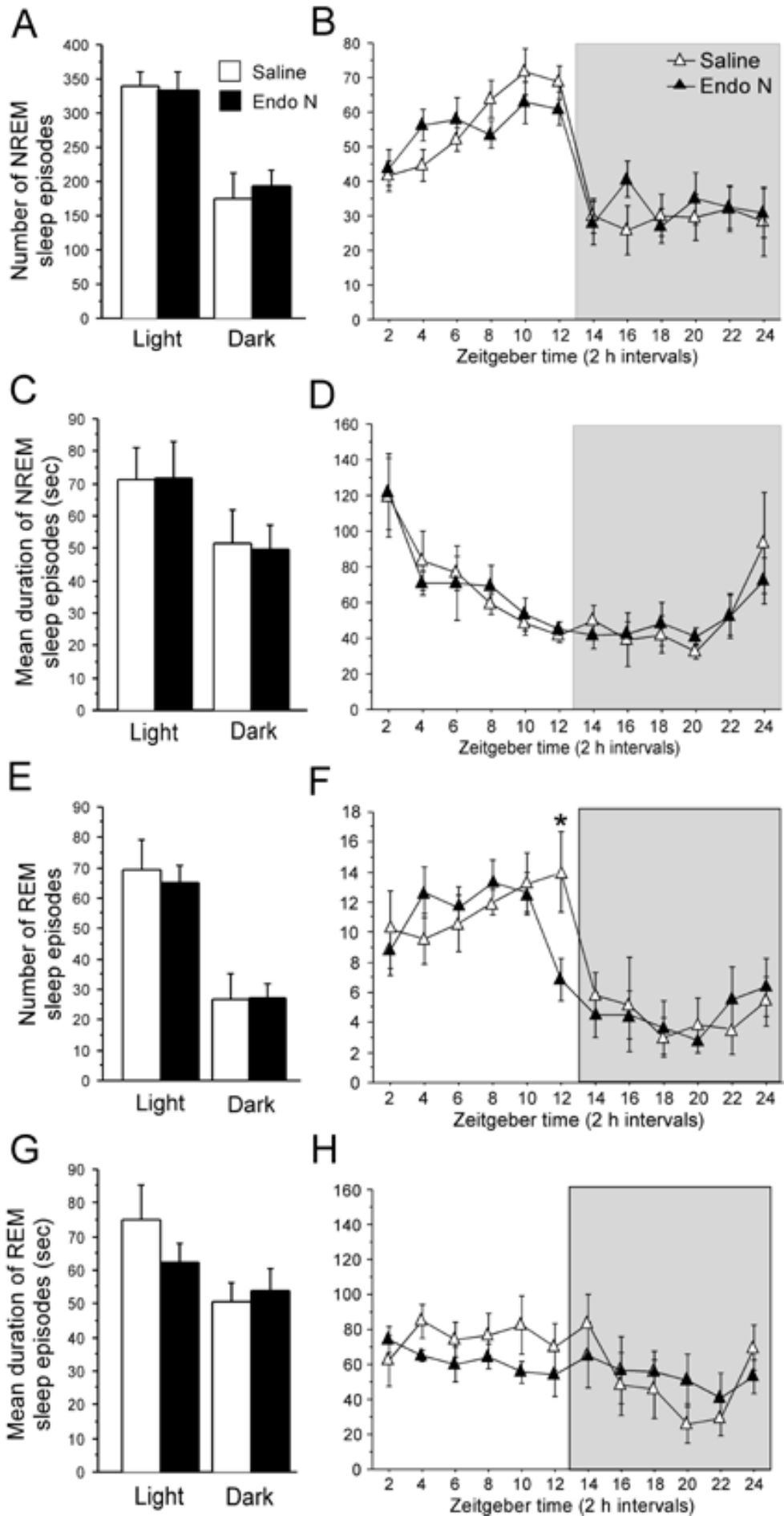

A
Number of NREM sleep episodes
Saline
Endo N
Light
Dark
B
Saline
Endo N
Zeitgeber time (2 h intervals)
C
Mean duration of NREM sleep episodes (sec)
Light
Dark
D
Zeitgeber time (2 h intervals)
E
Number of REM sleep episodes
Light
Dark
F
*
Zeitgeber time (2 h intervals)
G
Mean duration of REM sleep episodes (sec)
Light
Dark
H
Zeitgeber time (2 h intervals)

Figure 6

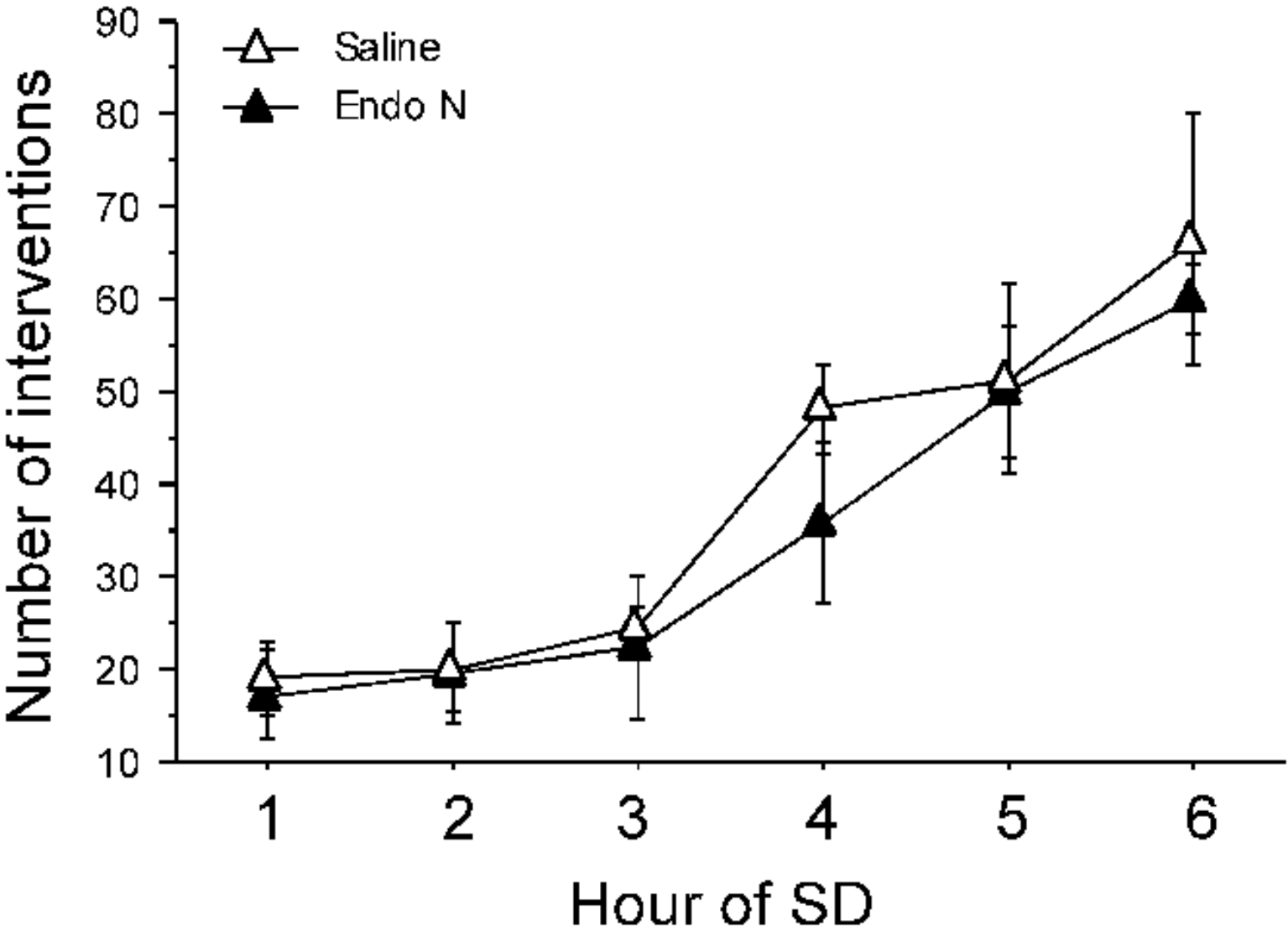

Saline
Endo N
Number of interventions
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
Hour of SD

Figure 7

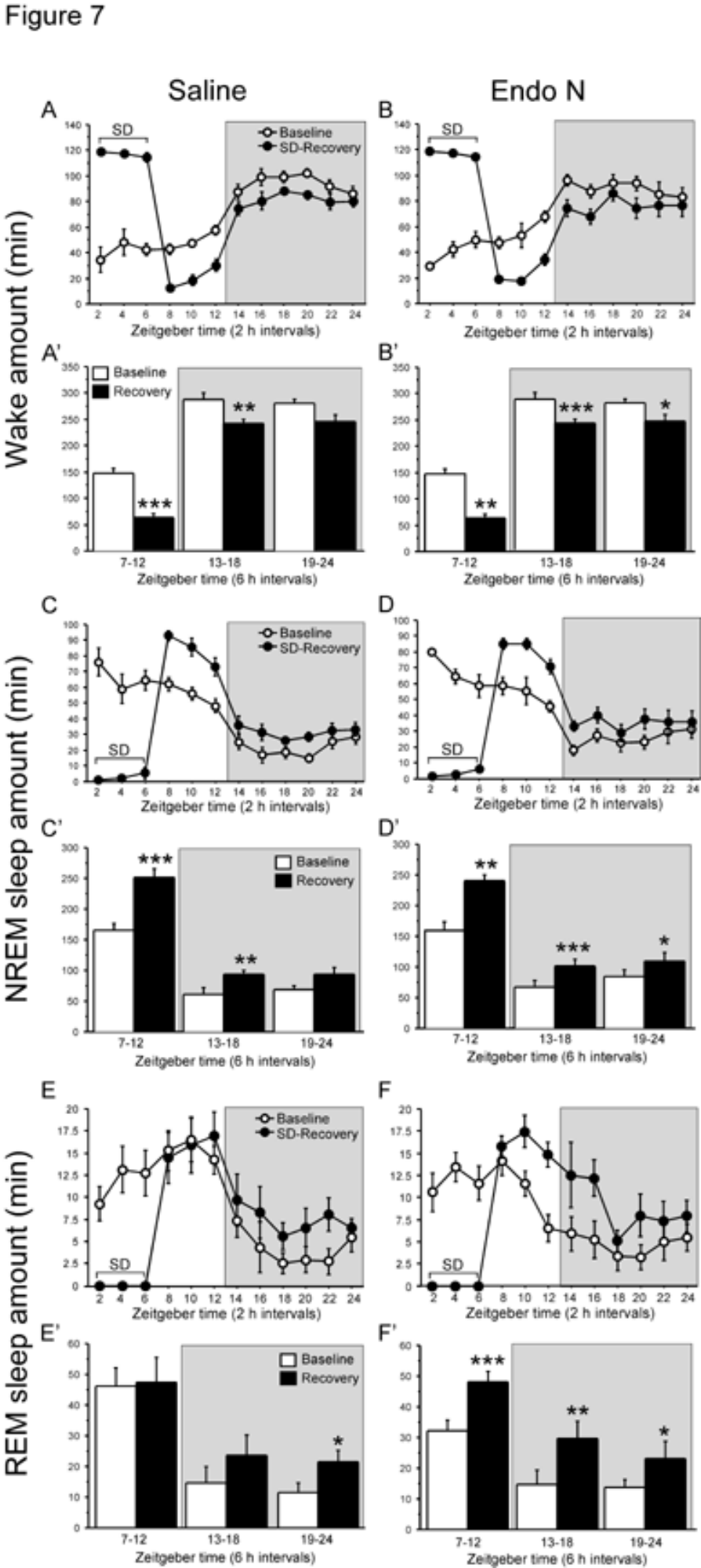

Saline
Endo N
Wake amount (min)
NREM sleep amount (min)
REM sleep amount (min)
A
B
A'
B'
C
D
C'
D'
E
F
E'
F'
SD
Baseline
SD-Recovery
Recovery
Zeitgeber time (2 h intervals)
Zeitgeber time (6 h intervals)
7-12
13-18
19-24
*
**
***

Figure 8

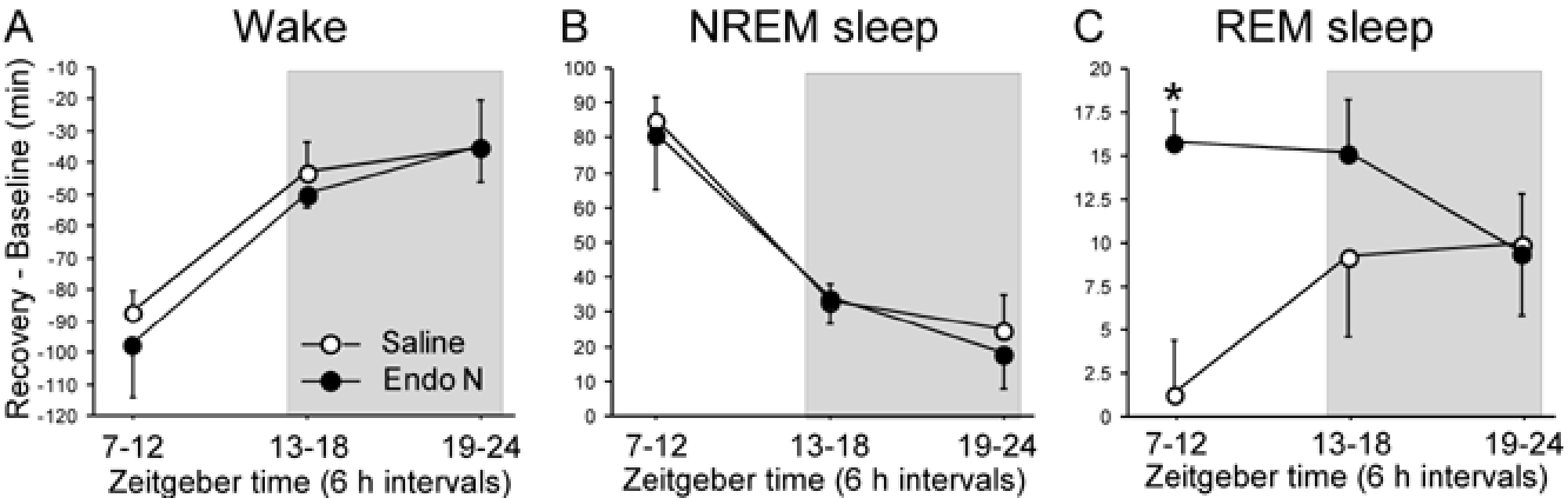

A
Wake
B
NREM sleep
C
REM sleep
Recovery - Baseline (min)
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
Saline
Endo N
100
90
80
70
60
50
40
30
20
10
0
20
17.5
15
12.5
10
7.5
5
2.5
0
*
7-12
13-18
19-24
Zeitgeber time (6 h intervals)

Table 1

Variable	Group	Condition	Zeitgeber time (6 h intervals)		
			7-12	13-18	19-24
NREM sleep episodes					
Number	Saline	Baseline	203±14	86±15	90±23
		Recovery	105±11**	110±11*	117±19
	Endo N	Baseline	177±13	95±12	98±14
		Recovery	95±5**	109±10	108±16
Mean duration	Saline	Baseline	50±5	46±10	56±11
		Recovery	150±18**	53±7	50±7
	Endo N	Baseline	56±7	45±9	54±7
		Recovery	155±13***	58±7*	67±12
REM sleep episodes					
Number	Saline	Baseline	39±5	14±6	13±4
		Recovery	36±2	19±4	18±4
	Endo N	Baseline	32±2	13±4	15±2
		Recovery	37±3	26±5***	19±2
Mean duration	Saline	Baseline	75±12	55±17	42±12
		Recovery	77±11	70±10**	76±10*
	Endo N	Baseline	60±7	59±9	48±9
		Recovery	82±10*	72±8	62±11

Supplementary Table 1

Antigen	Host	Source	Cat. #	Immunogen	Specificity
Adenosine deaminase	Rabbit	Chemicon	AB176 AB1815	Calf spleen adenosine deaminase	The antibody labels presumptive histaminergic neurons in the tuberomammillary nucleus, in agreement with Gerashchenko *et al.* (2004)
Cadherin	Mouse	Abcam	ab6528	Synthetic peptide corresponding to amino acids 889-912 of chicken n-cadherin	WB reveals a ~140 kDa band, corresponding to cadherin (Bae *et al.*, 2008; present study)
Choline acetyltransferase	Goat	Chemicon	AB144P	Human placental choline acetyltransferase	By WB, the antibody recognizes a band of 68 kDa, which corresponds to choline acetyltransferase (Matsumoto *et al.*, 2007)
Orexin B	Rabbit	Phoenix	H-003-32	The full 28 amino-acid orexin B peptide	Determined by competition radioimmunoassay (Technical Services, Phoenix)
PSA NCAM	Mouse	Developmental Studies Hybridoma Bank (Jessell and Dodd)	5A5	Embryonic rat spinal cord PSA NCAM	WB reveals a band of ~ 200 kDa, which corresponds to the sialylated isoform of NCAM (Acheson *et al.*, 1991; present study)
PSA NCAM	Mouse	Chemicon	MAB5324	Capsular polysaccharides of Meningococcus group B (strain 355)	By WB the antibody recognizes a band of 150 – 300 kDa, which corresponds to the sialylated isoform of NCAM (Rougon *et al.*, 1986; present study)
PSA NCAM	Mouse	Dr. T. Seki	mAB12E3	Fetal rat brain PSA NCAM	By WB the antibody recognizes a band of ~ 180 – 280 kDa, which corresponds to the sialylated isoform of NCAM (Seki & Arai, 1993)
Serotonin transporter	Guinea Pig	Chemicon	AB1772	Synthetic peptide corresponding to the C-terminus of the cloned rat serotonin transporter protein	The antibody labels serotonergic neurons in the dorsal raphe nucleus, in agreement with Steinbusch (1981)
Tyrosine hydroxylase	Rabbit	Pel-Freez	P40101	SDS-denatured, purified recombinant rat and bovine tyrosine hydroxylase	WB reveals a 60 – 62 kDa band, corresponding to tyrosine hydroxylase (Haycock, 1987)

IH, immunohistochemistry; WB, western blot.

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