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INSOMNIA FOLLOWING HYPOCRETIN2-SAPORIN LESIONS OF THE SUBSTANTIA NIGRA

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Abstract—The neuropeptide hypocretin, also known as orexin, has been implicated in waking since its deletion leads to the sleep disorder narcolepsy. Hypocretin neurons project to major arousal areas, and in an effort to determine which region is responsible for the changes in sleep–wake architecture we have developed the neurotoxin hypocretin2-saporin, which lesions hypocretin receptor bearing neurons. Here, in rats, we investigate the effects of hypocretin2-saporin lesions of the substantia nigra and ventral tegmental area in the regulation of sleep and wakefulness. Bilateral injection of hypocretin2-sap into both the ventral tegmental area and substantia nigra (92 and 184 ng/μl, 0.25 μl in the ventral tegmental area and 0.5 μl in the substantia nigra) or into the substantia nigra alone (184 ng/μl, 0.5 μl) produced insomnia. The insomnia seemed to be associated with a large increase in locomotion on days 4 and 6 postinjection, as hyperactivity and stereotypic movements were consistently observed on the video recordings in all lesioned rats. In these rats, a nearly complete loss of both tyrosine hydroxylase and neuron-specific nuclear protein (neuronal nuclei) immunoreactive cells in the substantia nigra as well as diminution of tyrosine hydroxylase-immunoreactive fibers in the caudate putamen was found. Following bilateral injection of hypocretin2-sap at a lower concentration (46 ng/μl, 0.25 μl in the ventral tegmental area and 0.5 μl in the substantia nigra), very little reduction in the number of tyrosine hydroxylase- and neuronal nuclei-immunoreactive neurons and only a temporary increase in wakefulness (17.4% increase during light-off period on day 6 postinjection) were observed. Ventral tegmental area lesions (184 ng/μl of hypocretin2-sap, 0.25 μl, bilateral injections) did not produce significant changes in sleep, although most of the tyrosine hydroxylase- and neuronal nuclei-immunoreactive neurons in the ventral tegmental area were destroyed. Insomnia following hypocretin2-sap lesions of the substantia nigra could be secondary to increased motor activity resulting from reduction of tonic inhibitory control by the substantia nigra. Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: EEG, electroencephalogram; EMG, electromyogram; HCRT2-sap, hypocretin2-saporin; MLR, mesencephalic locomotor region; MPTP, l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN, neuronal nuclei; NREM, non-rapid eye movement; PBS, phosphate-buffered saline; PPN, pedunculopontine tegmental nucleus; REM, rapid eye movement; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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The involvement of the substantia nigra (SN) and ventral tegmental area (VTA) in sleep regulation has been demonstrated in a number of studies (Lee et al., 2001; Honda et al., 1999; Maloney et al., 2002). However, the mechanisms of sleep regulation by these brain structures are not fully understood. There are at least two types of neurons in the SN and VTA that could play a role in sleep regulation: dopaminergic and GABAergic neurons. Regulation of sleep through dopamine neurotransmission is complex; that is, dopaminomimetics and the dopamine precursor L-dopa can promote sleep at low doses principally via D2-like presynaptic receptors, but can enhance wakefulness and suppress slow wave and rapid eye movement (REM) sleep at higher doses, probably via D1-like postsynaptic receptors (Rye, 2004; Monti, 1982). GABAergic neurons in the SN may be involved in REM sleep regulation via projections to the pedunculopontine tegmental nucleus (PPN) (Takakusaki et al., 2004b). In addition, these neurons could affect sleep indirectly through control of locomotor activity (Takakusaki et al., 2004a).

Lesion studies have been traditionally used to assess roles of different brain areas in sleep regulation. Dopaminergic neurons have been selectively lesioned by 6-hydroxydopamine (Srinivasan and Schmidt, 2004; Rodriguez et al., 2001), l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sedelis et al., 2000) or rotenone (Alam and Schmidt, 2004). Degeneration of dopaminergic neurons in the SN following administration of these neurotoxins produced symptoms of catalepsy and reduced locomotor activity in rodents. In the non-human primate, such lesions impaired daytime arousal and caused REM sleep to intrude into daytime naps (Rye, 2004). Insomnia and hyperactivity were observed in cats following non-selective *N*-methyl-D-aspartate bilateral lesions of the ventral mesencephalon that included the ventral mesencephalic reticular formation, retrorubral nucleus, VTA, SN pars compacta, reticulata and lateralis (Lai et al., 1999).

In the present study we used a neurotoxin, hypocretin2-saporin (HCRT2-sap), to lesion the SN and VTA. We previously used this neurotoxin to target neurons expressing the hypocretin-2 receptor (Blanco-Centurion et al., 2004; Gerashchenko et al., 2001a,b, 2003, 2004). A high expression of both the hypocretin 1 and 2 receptors is found in the SN (Hervieu et al., 2001; Marcus et al., 2001) and VTA (Lu et al., 2000; Marcus et al., 2001), and dopaminergic and non-dopaminergic neurons are excited by hypocretin in the VTA (Korotkova et al., 2003). Therefore,

we expected that the SN and VTA would be sensitive to HCRT2-sap.

EXPERIMENTAL PROCEDURES

Animals

The studies were conducted in accordance with the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as well as protocols approved by the local institutional committee on animal care and use. Twenty-four male Sprague–Dawley rats (270–370 g) were housed singly in Plexiglas cages with wood shavings, and with food and water available *ad libitum*. The rats were housed in a room where the temperature (21 °C) and the lights were controlled (7:00 a.m. to 7:00 p.m. lights on; 100 lux).

Drug groups and microinjection method

The following six groups were used: (1) rats injected with phosphate-buffered saline (PBS) into the VTA and SN ($n=5$); (2) rats injected with 184 ng/μl of HCRT2-sap into the VTA and PBS into the SN ($n=3$); (3) rats injected with 46 ng/μl of HCRT2-sap into the VTA and SN ($n=4$); (4) rats injected with 92 ng/μl of HCRT2-sap into the VTA and SN ($n=5$); (5) rats injected with 184 ng/μl of HCRT2-sap into the VTA and SN ($n=3$); (6) rats injected with 184 ng/μl of HCRT2-sap into the SN and PBS into the VTA ($n=4$). The solution of HCRT2-sap conjugate (Advanced Targeting Systems, San Diego, CA, USA) in PBS or PBS was delivered via a glass micropipette with a tip diameter of 20 μm by using a Picospritzer. The test substances were injected bilaterally into the VTA in a volume of 0.25 μl and bilaterally into the SN in a volume of 0.5 μl. After injection, the pipette was left in place for 5 min and then withdrawn slowly. The injections were made in the VTA at the following coordinates relative to bregma: AP=−5.3 mm; L=±0.6 mm; DV=7.5 mm below the dura, and in the SN at the following coordinates relative to bregma: AP=−5.3 mm; L=±2.4 mm; DV=7.7 mm below the dura (Paxinos and Watson, 1986). The concentrations of HCRT2-sap used were based on our previous studies on the effects of various concentrations of the HCRT2-sap in lesioning the HCRT-receptor containing cells in other brain areas (Blanco-Centurion et al., 2004; Gerashchenko et al., 2001a,b 2003, 2004). Similar concentrations of unconjugated saporin did not significantly lesion neurons in our previous study (Gerashchenko et al., 2003).

Surgical preparation

Rats were anesthetized by i.m. injection of a cocktail of acepromazine (0.75 mg/kg), xylazine (2.5 mg/kg) and ketamine (22 mg/kg). Using a stereotaxic apparatus, the rats were injected with the test substances (see drug groups above) and then implanted with electrodes to record the electroencephalogram (EEG) and electromyogram (EMG). Four miniature stainless steel screw electrodes were positioned in the skull to sit on the surface of the cortex and were used to record the EEG. Two miniature screws were inserted 2 mm on either side of the midline and 3 mm anterior to bregma (frontal cortex). The other two screws were located 2 mm on either side of the midline and 6 mm behind bregma (occipital cortex). The cortical EEG was recorded from two contralateral screws (frontal-occipital). To record muscle activity (EMG), two flexible multistranded wires were inserted in the nuchal muscles. The electrodes were placed in a plastic plug and secured onto the skull by using dental cement. After the surgery, the rats were returned to their home cages and EEG and EMG recordings were collected continuously for at least one week. After the experiment, the rats were transcardially perfused (after overdose of Nembutal) with 0.9% saline (50 ml) followed by 500 ml of phosphate-buffered 4% paraformaldehyde, pH 7.0 (formalin solution, cat. No. HT50-1-128; Sigma). The brains of formalin-perfused rats were postfixed overnight, equilibrated in 30% sucrose, and stored at 4 °C. All efforts were made to minimize animal suffering, and the smallest number of animals was used to produce reliable scientific data.

Analysis of sleep–wake states

EEG and EMG signals were recorded on a Grass polygraph and onto a Jaz disk using an analog-digital board (National Instruments, Austin, TX, USA). The EEG data were filtered at 70 Hz and 0.3 Hz and continuously sampled at 128 Hz. The 24-h EEG and EMG recordings obtained on the 2nd, 4th, 6th, and 14th day postinjection were scored manually on a computer (Icelus software, Mark Opp, Ann Arbor, MI, USA) in 12-s epochs for wakefulness, REM sleep and non-rapid eye movement (NREM) sleep by staff blind to the type of drug administered to the rats. Wakefulness was identified by the presence of desynchronized EEG and high EMG activity. NREM sleep consisted of high-amplitude slow waves together with a low EMG tone relative to waking. REM sleep was identified by the presence of desynchronized EEG and/or theta activity coupled with absence of EMG activity. The amount of time spent in wakefulness, NREM and REM sleep was determined for each hour. After the EEG data were scored, the code was broken to reveal the identity of each rat.

Immunohistochemistry

A one-in-five series of coronal sections were cut at 40 μm on a sliding microtome. Each set of coronal brain sections was incubated overnight at room temperature in mouse anti-tyrosine hydroxylase (TH) monoclonal antibody. After incubation, sections were rinsed in PBS and transferred into secondary antibody for 1 h (donkey anti-mouse IgG). After washing, the sections were incubated with an avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) for 1 h, washed again, and reacted in a 1% solution of 3,3′-diaminobenzidine with 0.01% hydrogen peroxide, as well as 0.05% nickel ammonium sulfate, to produce a gray–black stain. During the following night, sections were incubated with mouse anti-NeuN monoclonal antibody at room temperature, washed in PBS, and placed in the solution of the secondary antibody for 1 h (donkey anti-mouse IgG). The sections were then washed in PBS, incubated with biotin-conjugated alkaline phosphatase for 1 h (Vectastain ABC-AP Kit, cat. No. AK-5000; Vector Laboratories), washed again, and reacted in a working solution of Vector Red substrate (Vector® Red Alkaline Phosphatase Substrate Kit I, Cat. No. SK-5100; Vector Laboratories) to produce a red reaction product. Sections were mounted onto gelatin-subbed slides, dried, dehydrated in 100% ethanol, and delipidated in xylene. Slides were Nissl counterstained and coverslipped using Cytoseal™XYL mounting medium (Richard-Allan Scientific, Kalamazoo, MI, USA).

Antibodies

Mouse anti-TH monoclonal (1:15,000, cat. No. MAB318; Chemicon, Temecula, CA, USA), mouse anti-neuronal nuclei (NeuN) monoclonal (1:1000, cat. No. MAB377; Chemicon), and biotin-conjugated donkey anti-mouse IgG (1:500, cat. No. AP192B; Chemicon) antibodies were used.

Statistical analysis

Analysis of variance with Bonferroni *t*-tests correction was used to compare changes in sleep parameters (SYSTAT, Version 8.0, SPSS Inc., 1998). Statistical significance was evaluated at the $P<0.05$ level.