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EFFECT OF OREXIN-A ON DISCHARGE RATE OF RAT SUPRACHIASMATIC NUCLEUS NEURONS *IN VITRO**

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The suprachiasmatic nuclei (SCN) constitute the principal pacemaker of the circadian timing system in mammals. The generated rhythm is forwarded mostly through projections to various hypothalamic nuclei. On the other hand, the regulated processes feedback to the SCN. One of the possible feedback pathways is the orexinergic projection from the lateral hypothalamus. Orexins are recently identified neuropeptides with an overall facilitatory effect on waking behaviors. Orexinergic fibers are widely distributed throughout the brain and are also present in the SCN. In this study we examined the effect of orexin-A on the spontaneous activity of rat SCN cell *in vitro.* Neurons showed 2 different firing pattern (continuous-regular, intermittent-irregular). Orexin-A increased firing rate in both cell types at 10^{-8} M concentration, but caused a clear supression of neuronal activity at 10^{-7} M. Continuously firing neurons were less responsive than those firing intermittently. These results show that orexin-A may play a role in the modulation of the circadian pacemaker function. The neuropeptide might exert both direct, postsynaptic effects on SCN neurons and indirect, presynaptic effects on excitatory and inhibitory terminals. The dose-dependent modification of the firing rate indicate that the weight of these factors changes with the concentration of orexin-A.

Keywords: Circadian rhythms – cortical slices – orexin-A – SCN – single unit activity

INTRODUCTION

Orexins (also called hypocretins) are a recently isolated and identified family of hypothalamic neuropeptides. The two known forms, orexin-A (ORX-A) and orexin-B (ORX-B) are derived from the same 130-amino acid long precursor, called prepro-orexin. ORX-A contains 33 amino acids with two intrachain disulfide bonds, while ORX-B is a linear peptid of 28 amino acids [6, 12]. Two G protein-coupled receptors have been recognized for orexins, OX_1R and OX_2R. OX_1R is selective for orexin-A, while OX_2 is a non-selective receptor for both orexin-A and -B [12, 23]. Orexinergic neurons are located in the lateral hypothalamic area, and in the medial part of the

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zona incerta. Nerve terminals immunoreactive for orexins have been detected in the hypothalamus, thalamus, cerebral cortex, limbic system and in the brainstem monoaminergic structures [5]. These data suggest that orexins are likely to play a broad regulatory role in the CNS. Numerous studies have investigated the effect of ORX-A upon feeding, drinking, energy homeostasis, arousal and modulation of the sleep-wake cycle [7, 10, 12, 16, 22, 25, 29].

In mammals, the paired suprachiasmatic nuclei (SCN), located above the optic chiasm in the anterior hypothalamus have been identified as the primary pacemaker for circadian rhythms. They also have the ability to entrain the generated rhythms to environmental cues [19, 20]. The SCN receives both photic and non-photic inputs through three major pathways [19, 21]. Photic information reaches the SCN via the retinohypothalamic tract (RHT) that uses glutamate as transmitter and via the geniculohypothalamic tract (GHT) that releases NPY and GABA as transmitters [9, 21]. The principal non-photic innervation of the SCN arises from the median and dorsal raphe nuclei [15, 21]. It is supposed that environmental effects can synchronize and modulate the circadian pacemaker via these pathways. The suprachiasmatic nuclei also contain orexinergic terminals [5] and SCN neurons may express both OX_1 and OX_2 receptors. On the other hand, orexinergic neurons receive direct projection from the SCN [1]. The presence of orexinergic fibers and the supposed orexin-binding sites in the SCN suggest that these peptides may be involved in the modification of circadian rhythms.

Application of different neurotransmitters and peptides detected in the SCN have been found to modify neuronal firing rates in these nuclei *in vitro* and to induce behavioural changes *in vivo.* It has also been shown that ORX-A induces changes in arousal level and in behavioral states [10, 16], but no data are available on the effect of orexins on neuronal firing in the SCN. The aim of the present study was to investigate the effect of exogenously applied ORX-A on single unit activity of the rat SCN *in vitro.*

MATERIALS AND METHODS

Slice preparation and maintance

The experiments were performed on adult male SPRD rats (100–300 g; Human Gödöllő). Animals were housed under a 12–12 light-dark cycle (LD) for at least 7 days before the experiments. Zeitgeber time (ZT) 0 was defined as light-on at 08:00. Food and water was available *ad libitum.* On the day of the experiment at ZT 2, the animals were decapitated in deep nembutal anaesthesia (i.p. 35 mg/kg body mass) and the brain was quickly removed. A block containing the anterior hypothalamus and chiasma opticum was cut out, and coronal slices (500 μm) were prepared with vibrotome. Usually no more than one or two slices contained the small suprachiasmatic nuclei. Slices were incubated at room temperature in a carbogenated (95%

O_2–5% CO_2), HEPES-buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and its sodium salt, pH: 7.1–7.2) containing ACSF solution.

After 2 hours of incubation (ZT 2-4) slices were transferred into an interface type recording chamber continuously perfused with artificial cerebrospinal fluid (ACSF) at a rate of 2.8 ml/min. The composition of the ACSF was as follows (all values given in mM): 126 NaCl; 1.8 KCl; 1.25 K_2PO_4; 1.3 $MgSO_4$; 26 $NaHCO_3$; 2.4$CaCl_2$; 10 D-glucose; pH: 7.2–7.3 (Sigma). The ACSF solution was prewarmed to 32–33 °C and saturated with carbogene.

Electrophysiological recording

To record neuronal activity, glass extracellular electrodes (10–13 MW) were filled with 1 M NaCl. The signal was filtered and amplified (200 Hz–5 KHz) using an Axon-2B amplifier. Action potentials were recorded from the ventrolateral part of the SCN, at ZT 6-7. Single units (SUA) with a signal-to-noise ratio of 3 : 1 or more were only included in the experiments. Signals were digitized at 10 KHz and stored for off-line computer analysis on video tape using a VR 10A Digital Data Recorder (Instrutech Corp.).

Drug application

ORX-A (American Peptide Comp.) was dissolved in distilled water to yield a stock solution of 10 μM/l and stored at –18 °C. The stock solution was diluted with ACSF one hour before application. Drug administration started after a stable unit activity was found and a control period was recorded for at least 30 min. ORX-A was applied for 6 minutes into the perfusion solution in an end concentration of 10^{-8} M. Drug application was followed by a washing out period for 20 min and the treatment was repeated with a ten times higher dose (10^{-7} M).

Data acquisition and analysis

Data analysis was performed using a custom-written software. To isolate neuronal activity represented by discharges with reliable shapes, thus eliminating any artifacts or noises, the 3 least correlated parameters of spikes were measured: the amplitudes of the first and second peaks of the mostly biphasic discharges and the time elapsing between them [28]. These parameters determined a 3-dimensional vector. The end-points of those vectors belonging to similarly shaped spikes clustered close to each other. These clusters were manually delimited with ellipses and were supposed to correspond to the activity of individual neurons. For each unit, the average firing rate (Hz) and the coefficient of variation (var. coeff.) of the interspike intervals were determined. In addition, interval histograms (ISI) and autocorrelograms were calcu-

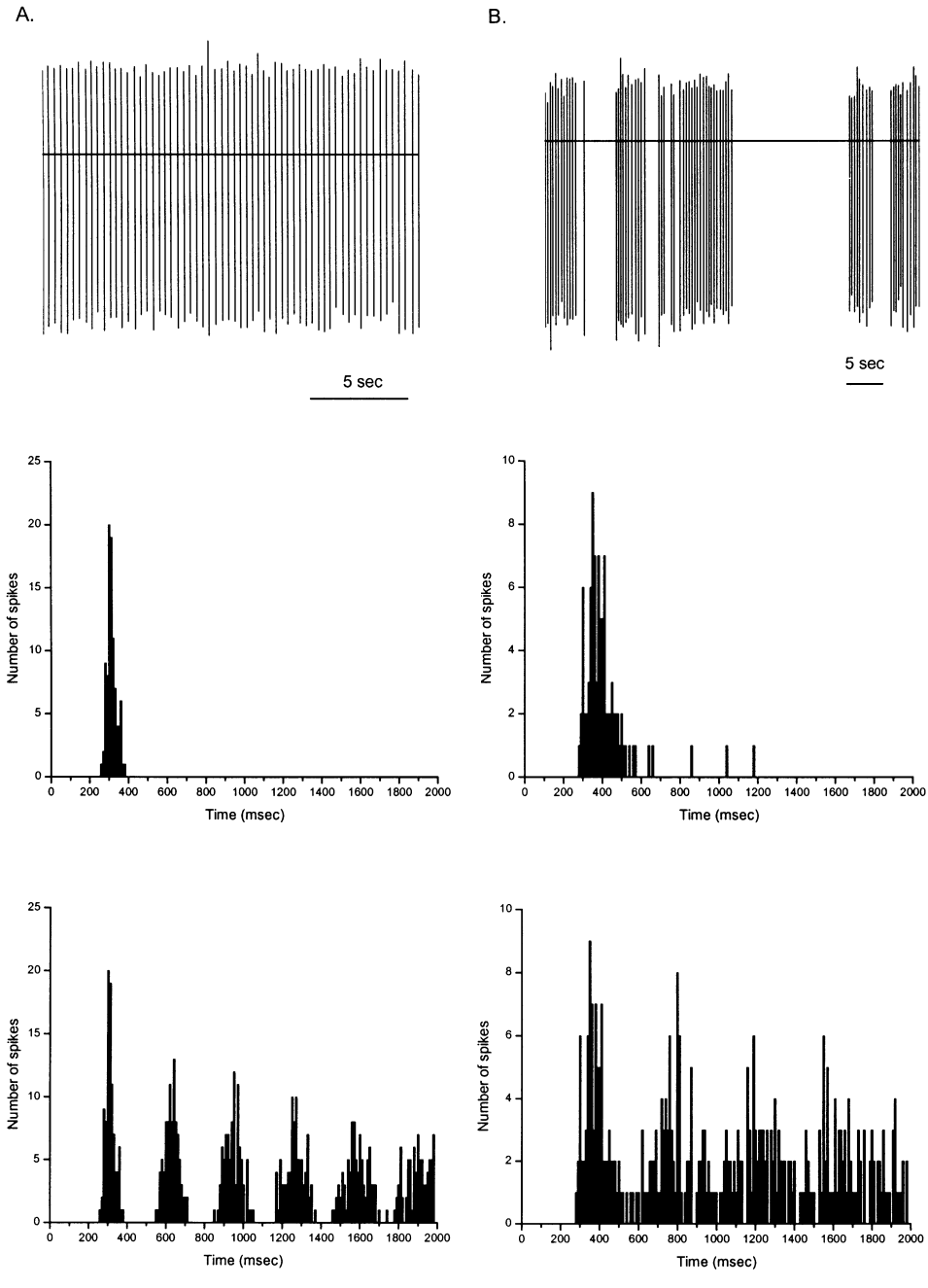

Fig. 1. Characteristics of the continuous-regular (A) and the intermittent-irregular (B) firing patterns. First row: single-unit activity of the spontaneously active neurons. Second row: interspike interval histograms. Third row: autocorrelograms

lated from the single unit activity to analyze the oscillatory properties of the recorded neurons. All calculations were carried out from 2-minute periods. One was selected just before the drug application (control) the other 8 minutes after the application started (treatment).

The recorded units were divided into 2 groups based on these parameters. Neurons with a var. coeff. less than 0.50 were designated as continuous-regular firing type. Neurons showing a higher variability in interval lengths, reflected in larger var. coeff. values, were called intermittent-irregular firing type. In the following, quantitative data are expressed as mean ±SEM. Due to the low number of the recorded neurons, statistical tests were only attempted for some of the changes.

RESULTS

Different types of the firing patterns

A total number of 9 neurons with stable spontaneous activity from 9 animals were included in the analysis. We placed the tip of the electorde on the surface of the area of the SCN and made 2–3 μm steps down until we found a spontaneously active neuron. If the activity remained stable for 30 minutes, the recording was started. Five cells (55.56%) showed continuous firing pattern, while 4 (44.44%) showed intermittent firing pattern. In each cases both the ISI and the autocorrelogram had a clear refractory period around the zero interval (i.e. a neuron cannot fire within a short interval after a spike). Regularly firing neurons had narrow ISI distribution and the autocorrelograms were characterized by 4–6 clear peaks at the integer multiplies of the primary interval length. In the irregularly firing neurons, the ISI reflected a wider distribution of intervals and only 1–2 peaks were seen in the autocorrelogram (Fig. 1). In general, when the frequency was lower, the regularity of the discharge rate was also low, but became higher as the frequency increased (Fig. 2).

Changes in firing rates

Cells showed an average basal firing rate of 3.62 ± 0.38 Hz (range 0.86–6.98 Hz). We found that 4 neurons out of the 9 recorded were clearly responsive to the drug application, while the remaining 5 gave a much weaker response (i.e. the change in the discharge rate relative to the baseline was less than 20%). The more regular discharge pattern was produced by a neuron, the less responsiveness we found.

At the lower concentration (10^{-8} M), ORX-A induced a slight activation in 4 of the 5 continuously firing cells; mean change: 7.03 ± 1.28% (range: 4.96 to 10.6%), and only one was supressed (–6.05%). Those cells, which had intermittent firing pattern ($n = 4$) were strongly activated; mean change: 52.63 ± 10.5% (range: 22.9–68.6%).

At the higher concentration (10^{-7} M) 4 continuously firing cells were suppressed; mean change: -3.19 ± 1.11% (range: 1.00 to 5.93%), and one cell was activated

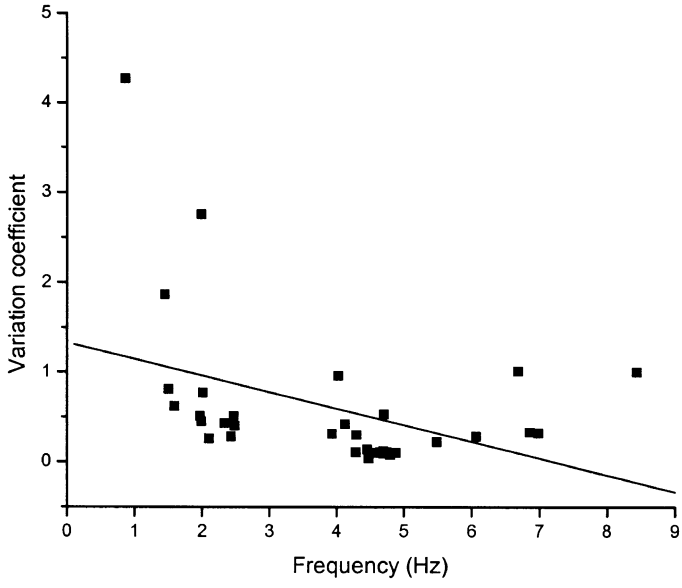

Fig. 2. Correlation between the firing rate and the variation coefficient. When the frequency was low, the firing pattern was irregular. At higher rates variation coefficient values revealed more regular neuronal activity. The frequency and the variation cefficient was significantly correlated at a level of $p < 0.02$

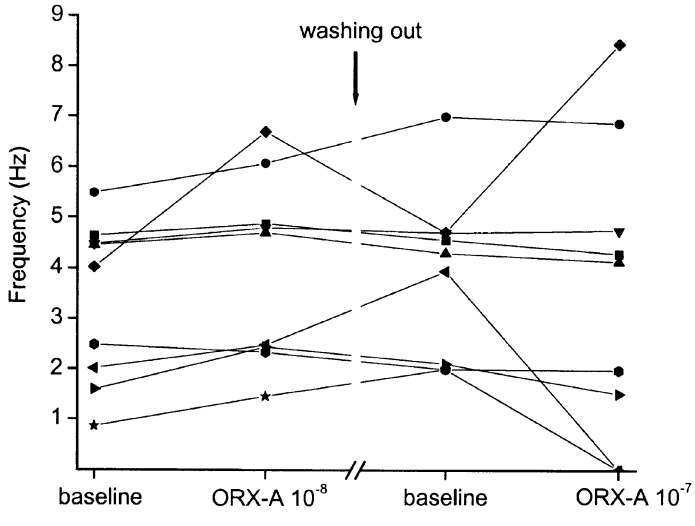

Fig. 3. Responses of single neurons to ORX-A

(1.07%). Three of those cells, which had intermittent firing pattern were supressed; mean change: $-76.2 \pm 23.8\%$ (range: 28.6 to 100) and one of these cells was activated (79.36%) (Fig. 3).

The distribution of activation and inhibition following the two treatments was significant at the level of $p < 0.05$ (Fischer's exact test).

DISCUSSION

The SCN is the principal pacemaker of the mammalian circadian timing system. Neurons of this nucleus have the ability to fire spontaneously at a low rate with different discharge patterns. However, this internally generated activity can be influenced by external signals. Both excitatory and inhibitory effects have been demonstrated on the spontaneous activity of the SCN neurons following the administration of different neuropeptides [3, 4]. However, no study has examined the effect of orexins on the SCN yet. The present study is the first description of the effect of orexin-A on the rat SCN *in vitro.*

In the present experiments two types of activity were found in the SCN at ZT 6–7: some cells displayed a continuous-regular firing type with a mean firing rate over 4 Hz, while others had a more irregular, intermittent firing type, with an average frequency of 2.6 Hz. There was a strong positive correlation between the discharge rate and the regularity of the firing. This finding is in agreement with earlier reports showing that the spontaneous activity has high variability below 3 Hz and becomes more and more regular as the firing rate increases [26]. In our sample, the highest frequency, produced by a continuously firing neuron, was 6.98 Hz, but the most regularly firing neurons were those, which had a firing rate between 4–5 Hz.

The suprachiasmatic nucleus entrains different circadian rhythms like the sleep-wake cycle and the behavior to environmental cues. Lots of transmitters and neuronal modulators are involved in this regulatory function [8, 9, 11, 12, 14, 15, 17, 18, 24, 30]. Among other peptides, orexins may be also included.

The exogenously applied neuropeptide Y was shown to have a clear inhibitory effect on the SCN [4], which effect could be blocked by neurotensin (NT) [3]. NT, applied alone, had a predominantly excitatory effect in more than 50% of the responsive cells, while 40% of the cells were not responsive to the NT treatment [3]. In our recordings the continuous-regular firing neurons were less responsive to the orexin-A application than those that fired irregularly. The correlation between the sensitivity of the neuron and the type of the firing pattern may be due to their different functions in the timekeeping system. It is also possible that those neurons, which are involved in the generation of the circadian rhythms (pacemaker neurons) may receive less input and thus may have fewer orexin receptors than those cells that have other functions.

In the present experiments orexin-A had a facilitatory effect on SCN neuronal firing in lower concentration, but a suppressive effect in higher concentration. On the basis of our data we could not decide whether ORX-A influenced neuronal firing rate

by acting directly on the spontaneously active SCN neurons or by regulating the release of other transmitters. In the medial and lateral hypothalamus ORX-A has a presynaptic facilitatory effect on both GABA- and glutamate-mediated transmission [27], and causes a selective excitation of GABAergic neurons in the substantia nigra [13]. Since both of these transmitter systems are present in the SCN it can be supposed that ORX-A acts also in these nuclei by influencing these systems. While glutamate is an exogenous transmitter in the SCN – terminals arise from the RHT –, GABA is present in most of the SCN cells [2]. We do not have any explanation for the concentration-dependency of the orexin effect. However, as ORX-A might have a direct effect on SCN neurons and might presynaptically influence both excitatory and inhibitory transmission, it can be supposed that the relative weight of these effects changes with the ORX-A concentration.

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