Acute Stimulation of Dissociated Cortical Neurons of Newborn Rats with Orexin A: Effect on the Network Activity

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Abstract— **Orexin A (OXA) and B are hypothalamic neuropeptides with recognized importance in the physiological regulation of various brain activities, including sleep/wakefulness, learning and memory, locomotion, autonomic control. Orexin activity is mediated by two types of receptors; OR1 binds OXA with higher affinity, while OR2 binds both ligands equally. There is a growing interest in OXA role in neurodegenerative diseases with respect to the non-motor symptoms such as sleep and attention disorders. Recent studies in Parkinson's patients found 40% lower concentration of OXA in the frontal cortex and 25% reduction in the cerebrospinal fluid. Both the number of orexinergic neurons in the hypothalamus and the levels of orexin in the cerebrospinal fluid are reduced by 72% in the end-stage in mouse model of Huntington's disease. Despite the extensive information about OXA expression and function in the nervous system of adults, data about the orexin system and its role in the functioning of developing neuronal networks are still insufficient. Neuronal cell cultures are often used as a model for brain inquiries; therefore, we undertook our study to investigate immunocytochemically the expression of OR1 in dissociated cortical neurons at various ages, and the acute effect of OXA application on the network activity of neurons, cultured on multi electrode arrays. Initially, control recordings were made after refreshing 50% (300 microliters) of the culture medium. After 2 hours, 300 microliters of medium were replaced again either with medium containing OXA (0.5-2 micromolar, n=8) or plain medium (n=5). Paired t-tests indicated a significant increase in the network activity after acute OXA application ($p<0.003$), but not after plain medium treatment ($p>0.32$), supporting the hypothesis that OXA stimulates neuronal activity in the cortex. These results indicate that potential drugs, based on OXA are attractive candidates for prevention and treatment of disorders associated with neuronal activity decline.**

Keywords— **orexin A, orexin receptor 1, neuronal network activity, multielectrode array, rat cortex.**

I. Introduction

Orexin A (OXA) and orexin B (OXB) are specific hypothalamic neuropeptides with recognized importance in the physiological regulation of various brain activities, including sleep/wakefulness, learning and memory, locomotion, autonomic control [1,2,3]. They have recently been implicated in neurodegeneration associated with narcolepsy, impaired learning and decreased memory abilities [4]. Recent studies in Parkinson's patients found 40% lower concentration of OXA in the frontal cortex and 25% reduction in the cerebrospinal fluid [5]. Both the number of orexinergic neurons in the hypothalamus and the levels of orexin in the cerebrospinal fluid are reduced by 72% in the end-stage in mouse model of Huntington's disease [6].

Orexin activity is mediated by two types of G-protein-coupled receptors which bind both orexins with varying selectivity; OR1 binds OXA with higher affinity, while OR2 binds both ligands equally [7].

Despite the extensive information about OXA expression and function in the nervous system of adults, data about the orexin system and its role in the functioning of developing neuronal networks are still insufficient.

Neuronal cell cultures are often used as a model for brain inquiries. We undertook our study to investigate immunocytochemically the expression of OR1 in dissociated cortical neurons at various ages, and the acute effect of OXA application on the network activity of neurons, cultured on multi electrode arrays.

II. Material and methods

A. Dissociated Cell Cultures

Cortical neurons were obtained from newborn Wistar rats. Under sterile conditions, the whole brain was removed and placed in a Petri dish with a RPMI-medium supplemented with extra glucose to a final concentration of 6.5 mg/ml. The meninges of the cortices were removed, and the striatum and the hippocampus were prepared free. The remaining cortices were collected in a tube with chemically defined R12 culture medium and trypsin for chemical dissociation. After removal of trypsin, 150 µl of soybean trypsin inhibitor and 125 ml of DNAse I (20.000 units, Life Technology) were added. It was followed by mechanical dissociation of the neurons into the solution, and the suspension was centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and the pallet of neurons was resuspended. The obtained neurons were plated and cultured on glass

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cover slips pre-coated with 20mg/ml poly-ethylene-imine (Fluka, Buchs, Switserland) for enhancement of the cell adhesion. Cells were kept in serum-free R12 medium under standard conditions of 37°C and 5% CO2 in air. An initial cell density approximately 3000 cells/mm^2 was used in all experiments. After one- or two-week incubation, cultures for detection of OR1 were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and processed immunocytochemically.

B. Electrophysiological Experiments

For the experiments, testing the acute effect of OXA on the network activity, neurons were cultured on multi electrode arrays. These are glass culture chambers with 60 electrodes (diameter of 10 or 30 μm) incorporated in the bottom. In all experiments, cultures were moved to the measurement set up one hour prior to recording to adapt to the conditions and we recorded baseline activity. Then we replaced 300 μl of the medium by 300 μl of fresh medium as a control for the effect of administration of an equal amount of OXA containing medium. After 2 hours recording, 300 microliters of medium were replaced again either with medium containing OXA (0.5-2 micromolar, n=8) or plain medium (n=5). We tested the differences in activity for statistical significance using a paired t-test. $P<0.05$ was considered significant.

C. Immunocytochemistry

The immunohistochemical staining procedure was performed according to the ABC (avidin-biotin-horseradish peroxidase) method. Briefly, a hydrogen peroxide (0.3% in absolute methanol for 30 min) was used to inactivate endogenous peroxidase. Appropriate washes in PBS followed this and subsequent treatments. Incubation in primary antibody goat anti-orexin receptor-1 IgG (AbD Serotec, Oxford, dilution 1:200) lasted for 20 h at room temperature and was followed by 2 h biotinilated donkey anti-goat IgG (1:500; Jackson ImmunoResearch, West in ABC complex (1:500; Vector Labs, Burlingame, CA, USA). Following rinsing, peroxidase activity was visualized using 2.4% SG substrate kit for peroxidase (Vector) in PBS for 5 min, at room temperature. Finally, the cultures were dehydrated in a graded series of alcohols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). Negative controls included incubation after antigen-antibody preabsorption with the native antigen, at 4 ^{o}C for 24 h. After immunostaining, the cultures were photographed with AxioCam MRC digital camera linked to a Zeiss Axioplan 2 research microscope. All digital images were matched for brightness and contrast in Adobe Photoshop 7.0 software.

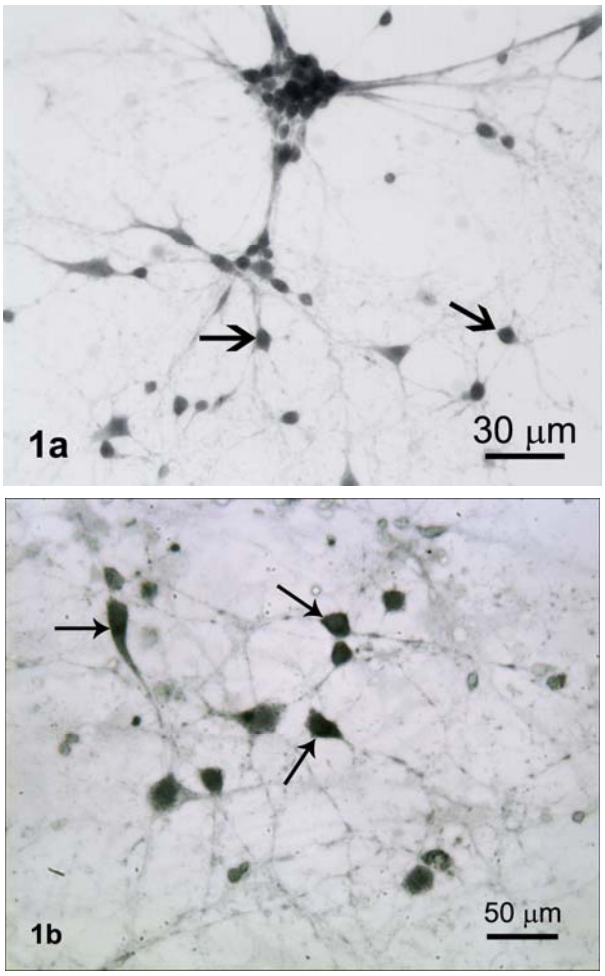

Fig. 1 Dark gray staining of OR1-immunoreactive neurons (arrows) in one-week (a) and two-week-old culture (b)

III. Results

No immunoreactivity for OR1 was detected in the cultures when the primary or secondary antibody was replaced with normal serum. The immunoreactivity was readily discernible at the light microscopic level by the presence of a dark-gray immunoreactive product. Neuronal structures were considered to be immunopositive when their staining was clearly stronger than that in the background.

Five cortical cultures for each incubating period were used for demonstration of OR1. Immunocytochemical labeling revealed that most of the neurons in one-week-old cultures were OR1-immunoreactive (IR). The reaction product was evenly distributed on the cellular surface and along neuronal outgrowth (Fig. 1a, b). This OR1-expressing popu-

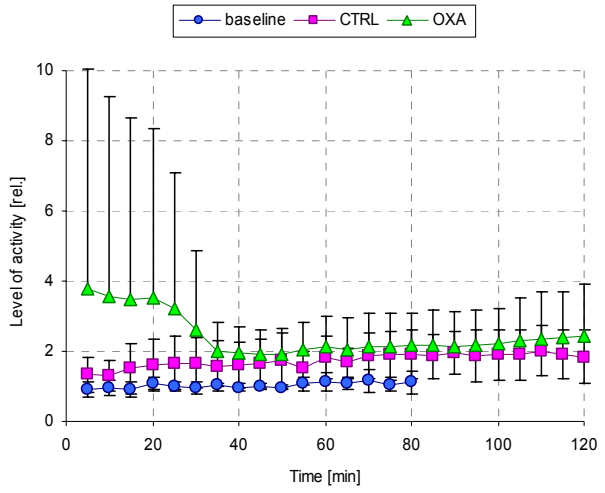

Fig. 2 Chart illustrating the significant increase in the network activity after OXA administration compared to the baseline and control experiment with plain medium

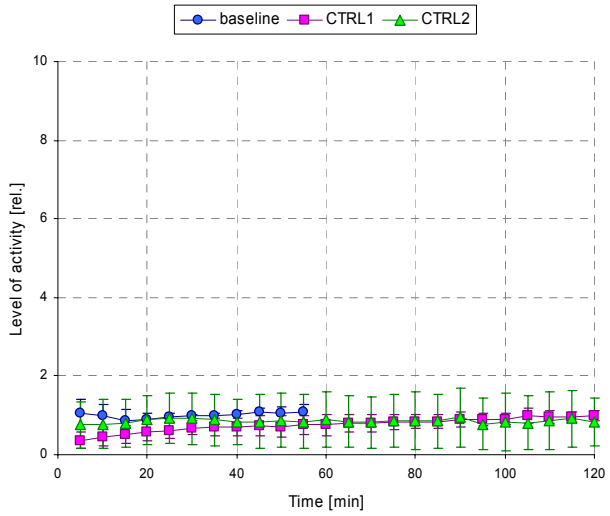

Fig. 3 Double control demonstrating a lack of significant change in the neuronal activity after two consecutive treatments of the culture with a plain medium

lation consisted of two types of cells: bipolar neurons with two major neurites arising from opposite poles of the cell body, and multipolar neurons with several major neurites emerging from a stellate-shaped soma. A fairly high density of OR1-IR neurons was observed in cultures incubated for two weeks.

In 13 experiments we evaluated the effect of acute OXA administration on electrical network activity. As a control, we first changed 300 µl of the medium with 300 µl fresh medium which did not have a significant effect on the activity. Then, we replaced 300 µl of the medium with a similar volume containing orexin. After both manipulations we recorded activity in the culture for 2 hours. Figure 2 compares the activity after OXA administration to that after control. OXA increased the network activity particularly during the first half an hour. Figure 3 demonstrates that there was no significant change in the network activity after two consecutive administrations of a plain medium.

Paired t-tests indicated a significant increase in the network activity after acute OXA application ($p<0.003$), but not after plain medium treatment ($p>0.32$), supporting the hypothesis that OXA stimulates neuronal activity in the cortex.

IV. Discussion

Our study clearly demonstrates that dissociated cortical neurons are well equipped with OR1 and the acute application of the neurotransmitter OXA enhances their electrical activity. Analogous effect was shown with recent *in vivo* studies on genetically modulated animal models, where the loss of orexinergic neurons led to similar behavioral characteristics as humans with neurodegenerative diseases [8]. In addition, replacement of orexin in those animal models reversed some, if not most of the deficits observed [9]. Moreover, the permanent ischemia after brain injury dramatically increases the OR1 mRNA and protein expression, suggesting that orexins, and particularly OXA, play a role in neuronal survival/death [10]. This is in consent with our finding that in culturing conditions neurons express OR1 at early stages of development and OXA stimulation significantly increases neuronal activity.

V. Conclusions

Dissociated cortical neurons of the newborn rats express OR1 and the acute application of OXA significantly increases the network activity. These results indicate that potential drugs, based on OXA are attractive candidates for prevention and treatment of disorders associated with neuronal activity decline.

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