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# Cytosolic calcium elevation induced by orexin/hypocretin in granule cell domain cells of the rat cochlear nucleus in vitro

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ABSTRACT

Using rat brain slice preparations, we examined the effect of orexin on cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) in the granule cell domain (GCD) cells of the cochlear nucleus that carry non-auditory information to the dorsal cochlear nucleus. Application of orexin concentration-dependently increased $[Ca^{2+}]_i$, and in two thirds of GCD cells these increases persisted in the presence of tetrodotoxin. There was no significant difference between the dose–response curve for orexin-A and that for orexin-B. Extracellular $Ca^{2+}$ removal abolished the $[Ca^{2+}]_i$ elevation induced by orexin-B, whereas depletion of intracellular $Ca^{2+}$ stores had no effect. The orexin-B-induced elevation of $[Ca^{2+}]_i$ was not blocked by inhibitors of reverse-mode $Na^+/Ca^{2+}$ exchanger (NCX) and nonselective cation channel, whereas it was blocked by lowering the extracellular $Na^+$ or by applying inhibitors of forward-mode NCX and voltage-gated R- and T-type $Ca^{2+}$ channels. The ORX-B-induced increase in $[Ca^{2+}]_i$ was also blocked by inhibitors of adenylcyclase (AC) and protein kinase A (PKA), but not by inhibitors of phosphatidylcholine-specific and phosphatidylinositol-specific phospholipase C. In electrophysiological experiments using whole-cell patch clamp recordings, half of GCD cells were depolarized by orexin-B, and the depolarization was abolished by a forward-mode NCX inhibitor. These results suggest that orexin increases $[Ca^{2+}]_i$ postsynaptically via orexin 2 receptors, and the increase in $[Ca^{2+}]_i$ is induced via the AC–PKA–forward-mode NCX-membrane depolarization-mediated activation of voltage-gated R- and T-type $Ca^{2+}$ channels. The results further support the hypothesis that the orexin system participates in integrating neural systems that are involved in arousal, sensory processing, energy homeostasis and autonomic function.



## 1. Introduction

Orexin-A (ORX-A) and orexin-B (ORX-B), also called hypocretin-1 and hypocretin-2, respectively, are novel neuropeptides that are synthesized in the perifornical region of the lateral hypothalamic area (LHA). ORX-A and ORX-B bind to ORX 1 ($OX_1$) receptors and ORX 2 ($OX_2$) receptors that belong to the G protein-coupled receptor superfamily [33]. $OX_1$ receptors have a higher affinity for ORX-A than for ORX-B, whereas $OX_2$ receptors have a similar affinity for both ORX-A and ORX-B. The nerve terminals of ORX neurons from the perifornical region of the LHA are distributed throughout almost the entire brain, including the cortex, limbic system, hypothalamus and brainstem [6,22,26,30]. In accordance with the distribution of nerve terminals, $OX_1$ and/or $OX_2$ receptors are also found in these brain regions [5,10,20,21,41]. These widespread distributions of ORX nerve terminals and receptors in the brain suggest multifunctional roles for the ORX system. Indeed, potential roles for ORX-A and ORX-B have already been demonstrated; these include the regulation of arousal, sensory processing, energy homeostasis, and autonomic functions [27,36].

The gateway for neural processing in the ascending auditory system is the cochlear nucleus. This nucleus is divided into two parts: a magnocellular core and a microneuronal shell [7,32]. The microneuronal shell is mainly situated over the medial, dorsal and lateral surface of the ventral cochlear nucleus and expands into layer II of the dorsal cochlear nucleus [23,24,32,45]. The microneuronal shell includes three types of cells – granule, unipolar brush and chestnut cells – and it is sometimes referred to as the granule cell domain (GCD) due to the abundance of granule cells [7,32,45]. The GCD receives non-auditory inputs rather than rapidly conducted auditory inputs, and it sends its output to the dorsal cochlear nucleus [7,32,45]. The non-auditory inputs include vestibular signals concerning head position and somatic proprioceptive signals that indicate neck muscle position and tension. The level of arousal is also one of the non-auditory inputs to the GCD [32]. Indeed, spontaneous and evoked unitary firing of the cochlear nucleus exhibit changes closely related to stages of sleep and wakefulness [28]. ORX neurons in the LHA also change their discharge rate across the sleep-waking cycle; they increase firing during and preceding active waking, and virtually cease firing during sleep [19]. ORX-

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immunoreactive nerve terminals project to the cochlear nucleus [9,22,26,30], and neurons in the cochlear nucleus express $OX_1$ and $OX_2$ receptors [5,9,10,21]; this suggests a close relationship between the ORX system and auditory sensory processing.

Alterations of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) have been shown to regulate many neuronal functions, such as neuronal excitability, transmitter release, gene expression, neuronal plasticity, cell survival, apoptosis and enzyme activity [4,38]. Sakurai et al. [33] were the first to discover that ORX induces a transient increase in $[Ca^{2+}]_i$ in Chinese hamster ovary (CHO) cells which recombinantly express human ORX receptors. Subsequent studies in rodents further demonstrated that ORX elevates $[Ca^{2+}]_i$ in neurons in the various brain regions to which ORX fibers project and in which ORX receptors are expressed [11,17,42,43,44]. Thus, it seems likely that $[Ca^{2+}]_i$ in GCD cells of the cochlear nucleus may be also elevated by ORX via ORX receptors. However, the effects of ORX on $[Ca^{2+}]_i$ of GCD cells have not been described. Therefore, the aim of the present study was to examine the effects of ORX on $[Ca^{2+}]_i$ in GCD cells, using rat brain slice preparations. To investigate the electrophysiological effects of ORX on GCD cells, whole-cell patch clamp recordings were also made.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats, 12–16 days old, were used (Sankyo Lab., Shizuoka, Japan). The rats were housed with their mothers in a light-controlled room (light on: 06:00–18:00) at a temperature of $23 \pm 1\,^{\circ}C$ for several days prior to the experiments. Food and water were available ad libitum. The animals and experimental procedures used were approved by the Institutional Animal Care and Use Committee of the University of Toyama.

### 2.2. Slice preparation

After sevoflurane anesthesia, the rats were decapitated and their brains were rapidly removed from the skull. The brains were then submerged in ice cold, oxygenated (95% $O_2$–5% $CO_2$) standard artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, KCl 3, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $KH_2PO_4$ 1.25, $NaHCO_3$ 26 and glucose 10, with a pH of 7.4. Frontal brainstem slices, 250 μm thick, were cut by a microslicer (ZERO 1, Dosaka EM, Kyoto, Japan). The GCD of the cochlear nucleus was identified on the basis of its anatomical location and shape. Two or three slices including the GCD were selected from each animal, and the cerebellum dorsal to the cochlear nucleus was removed with a scalpel. The slices were further cut along the midline with the scalpel, such that two slices (each including the left and right GCD) were obtained. The slices containing the GCD were then preincubated in a chamber with oxygenated ACSF for at least 1 h at room temperature.

### 2.3. $[Ca^{2+}]_i$ imaging

After preincubation, slices were transferred on a membrane filter, which was placed on a culture dish, and submerged in ACSF containing the calcium indicator fura-2 acetoxyl methyl (fura-2 AM, 10 μM) and pluronic F127 (0.0125%). The culture dishes were then placed in a tightly sealed pressure box filled with 95% $O_2$–5% $CO_2$ at a pressure of 70 kPa, at room temperature for 1 h. This procedure enabled GCD cells of the cochlear nucleus to become loaded with fura-2. The slices were then transferred to a recording chamber, which was attached to the stage of an upright microscope (BX51WI, Olympus, Tokyo, Japan), and perfused with oxygenated ACSF at 1 ml/min and 34 °C. Fura-2 fluorescence (510 nm) was excited by means of a xenon lamp (U-LH75XEAPO, Olympus, Tokyo, Japan) through a filter exchanger with filters for 340 and 380 nm (C8214, Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence of the loaded cells was imaged by a high-speed cooled digital charge coupled device (CCD) camera (C6790, Hamamatsu Photonics, Hamamatsu, Japan) with the output digitized to a resolution of $512 \times 483$ pixels. The images had exposure times of 100–200 ms and were collected either every 10 s for a period of about 8 min after ORX application or every 20 s during the interval between ORX effects. The images were then stored on the hard disk of a PC computer via the data acquisition equipment (C7746, Hamamatsu Photonics, Hamamatsu, Japan). Regions of interest were selected to encompass individual 7–10 individual cells in the GCD in each slice. Fluorescence data were analyzed on-line and off-line on the computer using data analysis software (Aquacosmos, Hamamatsu Photonics, Hamamatsu, Japan), and displayed on a computer screen. $[Ca^{2+}]_i$ was first expressed as a ratio ($F_{340}/F_{380}$) of fluorescence intensity excited at 340 nm to that excited at 380 nm. The $F_{340}/F_{380}$ ratio was then normalized by the mean value of the $F_{340}/F_{380}$ ratio during a 4-min period immediately prior to the first application of ORX in standard ACSF. ORX-B (100 nM) was first applied in standard ACSF, and when the number of cells responsive to ORX-B in each slice was less than or equal to 2, the slice was no longer used for further experiments. Each $[Ca^{2+}]_i$ response to ORX was calculated as the difference between the average value of normalized $F_{340}/F_{380}$ ratio during the 2 min immediately prior to each application of ORX and the maximum value of the 1-min moving averages of normalized $F_{340}/F_{380}$ ratio that were obtained over 8 min after the application of ORX.

### 2.4. Whole-cell patch clamp recording

After preincubation, slices were transferred to a whole-cell patch clamp recording chamber that was fixed to the stage of an upright microscope (BX-50WI, Olympus, Tokyo, Japan). The recording chamber was perfused with oxygenated standard ACSF at 1 ml/min and 34 °C. GCD cells were visualized on a television screen via an infrared CCD camera (C2741-79, Hamamatsu Photonics, Hamamatsu, Japan) and a real-time digital video microscopy processor (XL-20, Olympus, Tokyo, Japan). Electrodes were filled with a standard internal solution that contained (in mM): K-gluconate 120, KCl 20, HEPES 10, $MgCl_2$ 2.0, $CaCl_2$ 0.5, EGTA 1.0, Na-ATP 4.6, and Na-GTP 0.4, with pH adjusted to 7.3 with KOH. The electrode resistances ranged from 5 to 9 MΩ. Cells were recorded in "I-clamp normal" current clamp mode using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, USA). An Ag/AgCl reference electrode was placed near the intermediate position between the inlet and outlet of the recording chamber. Only a single cell was recorded from each slice. Membrane potentials recorded via the electrodes were fed into the amplifier. Series resistance compensation and capacitive compensation were performed to the greatest extent possible by the amplifier. The output of the amplifier was digitized using an A/D converter board (Digidata 1200, Axon Instruments, Union City, USA) with a sampling rate of 10 kHz, and recorded on a hard disk via data acquisition and analysis software (pCLAMP 8, Axon Instruments, Union City, USA). Membrane potentials were low-pass filtered at 5 kHz. Whole-cell liquid junction potentials were calculated to be about −13 mV for the internal solution, and membrane potentials were not corrected. In current clamp mode, membrane potentials less than −60 mV were set to about −60 mV by current injection.

### 2.5. Reagents

All drug-containing solutions were freshly prepared for the experiments. ORX-A and ORX-B (Peptide Institute, Osaka, Japan), tetrodotoxin (TTX, Wako Pure Chemical Industries, Tokyo,