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RAPID REPORT

OPPOSITE EFFECTS OF NORADRENALINE AND ACETYLCHOLINE UPON HYPOCRETIN/OREXIN VERSUS MELANIN CONCENTRATING HORMONE NEURONS IN RAT HYPOTHALAMIC SLICES

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Abstract—Hypocretin/orexin (Hcrt/Orx) and melanin concentrating hormone (MCH) are peptides contained in overlapping cell groups of the lateral hypothalamus and commonly involved in regulating sleep–wake states and energy balance, though likely in different ways. To see if these neurons are similarly or differentially modulated by neurotransmitters of the major brainstem arousal systems, the effects of noradrenaline (NA) and carbachol, a cholinergic agonist, were examined on identified Hcrt/Orx and MCH neurons in rat hypothalamic slices. Whereas both agonists depolarized and excited Hcrt/Orx neurons, they both hyperpolarized MCH neurons by direct postsynaptic actions. According to the activity profiles of the noradrenergic locus coeruleus and cholinergic pontomesencephalic neurons across the sleep–waking cycle, the Hcrt/Orx neurons would be excited by NA and acetylcholine (ACh) and thus active during arousal, whereas the MCH neurons would be inhibited by NA and ACh and thus inactive during arousal while disinhibited and possibly active during slow wave sleep. According to the present pharmacological results, Hcrt/Orx neurons may thus stimulate arousal in tandem with other arousal systems, whereas MCH neurons may function in opposition with other arousal systems and thus potentially dampen arousal to promote sleep.

Key words: sleep–wake states, REM sleep, arousal, energy metabolism, electrophysiology, *in vitro*.

Since very early studies, the posterior lateral hypothalamus has been known to play a critical role in maintaining wakefulness (see for review (Jones, 2000). Most recently, magnocellular neurons in this area have been found to contain the peptide hypocretin or orexin (Hcrt/Orx) that was discovered to be the peptide or ligand to the receptor lacking in narcolepsy (Peyron et al., 1998; Chemelli et al., 1999; Lin et al., 1999; Thannickal et al., 2000; Hara et al., 2001). This syndrome is characterized by an inability to maintain wakefulness with sudden intrusions of sleep, particularly rapid eye movement sleep (REMS) and its associated loss of muscle tonus. In Hcrt/Orx knockout mice, the syndrome is also associated with obesity despite hypophagia due to a decrease in basal metabolic rate. Hcrt/Orx-containing neurons thus apparently stimulate arousal and metabolism in parallel, likely through their common influence upon arousal systems and the sympathetic nervous system carried by diffuse projections to the forebrain, brainstem and spinal cord (Peyron et al., 1998). As evidenced by c-Fos expression during sleep deprivation, these neurons are most active during waking when they would act upon their target neurons (Estabrooke et al., 2001). *In vitro* electrophysiological studies show that Hcrt/Orx excites cells of all the major arousal systems (Jones, 2003), including importantly the noradrenergic locus coeruleus neurons (Horvath et al., 1999) and the cholinergic pontomesencephalic neurons (Burlet et al., 2002). The Hcrt/Orx neurons would thus be expected to act in tandem with the major arousal systems to stimulate waking and appropriate autonomic responses.

Another peptide, melanin concentrating hormone (MCH) is also located in magnocellular neurons of the lateral hypothalamus (Bittencourt et al., 1992). Like the Hcrt/Orx neurons, MCH neurons also project widely through the CNS including the forebrain, brainstem and spinal cord and onto neurons of the arousal systems (Bittencourt et al., 1992). Yet the role and actions of MCH appear to be very different from those of Hcrt/Orx. First, MCH knockout mice do not show narcolepsy, in contrast to Hcrt/Orx knockout mice, and are lean due to an increase in basal metabolic rate as well as hypophagia (Shimada et al., 1998). Second, MCH has not been shown to excite, as has Hcrt/Orx, the neurons of the arousal systems. Third, MCH appears to depress, whereas Hcrt/Orx appears to enhance, synaptic activity by its actions upon calcium channels (van den Pol et al., 1998; Gao and van den Pol, 2001). Fourth, c-Fos expression occurs in MCH neurons with sleep recovery, whereas it occurs in Hcrt/Orx neurons with sleep deprivation (Modirrousta et al., 2003; Verret et al., 2003). These results suggest that MCH and Hcrt/Orx

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Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; ADP, after-depolarization; GFP, green fluorescent protein; Hcrt/Orx, hypocretin/orexin; IR, immunoreactive; LTS, low-threshold spike; MCH, melanin concentrating hormone; NA, noradrenaline; Nb, neurobiotin; REMS, rapid eye movement sleep; SWS, slow wave sleep; TTX, tetrodotoxin.

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may play different roles in sleep–wake states and energy metabolism.

Given such potentially different roles, MCH and Hcrt/Orx may be differentially modulated by the neurotransmitters of the arousal systems, particularly noradrenaline (NA) and acetylcholine (ACh). To test this hypothesis, we have examined the actions of NA and carbachol, a cholinergic agonist, upon previously characterized Hcrt/Orx versus MCH neurons in rat hypothalamic slices (Eggermann et al., 2003).

EXPERIMENTAL PROCEDURES

Brain slices were obtained from young (15–28 days) Sprague–Dawley rats reared at the Centre Medical Universitaire and animal facility of the Geneva Medical Center and treated according to the Swiss Federal Veterinary Office whose standards meet those of the Association for Assessment and Accreditation of Animal Care International. A minimal number of animals (34) were employed and killed rapidly by decapitation to minimize suffering. Coronal slices (250–300 μm thick) were cut on a vibratome through the posterior tuberal hypothalamus at the level where Hcrt/Orx and MCH-containing neurons are co-distributed through the perifornical region. They were incubated at room temperature in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 130, KCl 5, KH_2PO_4 1.25, $MgSO_4$ 1.3, $NaHCO_3$ 20, glucose 10 and $CaCl_2$ 2.4, bubbled with 95% O_2 and 5% CO_2. Individual slices were transferred to a thermoregulated (32 °C) recording chamber where they were immersed and continuously superfused at 4–5 ml/min with ACSF. The chamber was mounted on an upright microscope (Zeiss Axioskop, Oberkochen, Germany) equipped with an infrared camera for visualizing and patching cells.

Whole-cell recordings were performed with patch pipettes (7–10 MΩ) that were pulled on a DMZ universal puller (Zeitz-Instrumente, Munich, Germany) from borosilicate glass capillaries (GC150F-10; Clark Instruments, Edenbridge, UK). The pipettes were filled with an internal solution containing (in mM): $KMeSO_4$ 126, KCl 4, $MgCl_2$ 5, BAPTA 0.1, HEPES 10, phosphocreatine 8, ATP 3, GTP 0.1, pH 7.3, 285–300 mOsm. In some cases, neurobiotin (Nb; 0.2%; Vector Laboratories, Burlingame, CA, USA) was added to the intra-pipette solution to label cells for identification after recording. Whole-cell recording was performed in the current clamp mode using an Axopatch 200B (Axon Instruments, Foster City, CA, USA). The membrane potential values were not compensated for junction potential (estimated at −9.6 mV).

Drugs, including NA and carbachol (Schnelldorf, Germany) and tetrodotoxin (TTX; Latoxan, Rosans, France), were applied in the bath. Synaptic blockade was achieved by lowering calcium and increasing magnesium (0.1 mM Ca^{2+}, 10 mM Mg^{2+}).

Following recording and labeling of neurons, the slices were fixed by immersion in a 3% paraformaldehyde solution for 2–12 h. In one previously published series (Eggermann et al., 2003), slices were frozen and cut in 45 μm thick sections and processed for triple immunostaining as previously described for identification of Nb-labeled neurons as Orx vs. MCH-immunopositive. In the series presented here, whole slices (250–300 μm thick) were processed employing 0.3% Triton (in Tris NaCl, pH 7.4), for dual-immunostaining for Nb and Orx or MCH, depending upon the electrophysiological characteristics of the recorded neuron. The slices were incubated overnight at room temperature in a solution with 1) anti-Orexin A antiserum from goat (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 2) anti-MCH antiserum from rabbit (1:200; Brischoux et al., 2001; kindly supplied by P. Y. Risold and D. Fellmann, Besançon, France). They were subsequently incubated for 3 h at room temperature in a solution containing 1) Cy3-conjugated anti-goat antiserum (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for Orx or 2) Alexa Fluor 488-conjugated anti-rabbit antiserum (1:200; Molecular Probes, Leiden, Netherlands) for MCH together with 3) AMCA-conjugated streptavidin (1:100; Jackson ImmunoResearch Laboratories) for Nb.

RESULTS

Neurons were recorded in slices from the tuberal hypothalamic region where a maximum number of Hcrt/Orx- and MCH-immunoreactive (IR) neurons are co-distributed in an overlapping manner within the perifornical region (Fig. 1A, B). Under infrared illumination, multipolar or fusiform, large-sized neurons (diameter >20 μm) were sought in the region overlying the fornix. Using previously established electrophysiological characteristics (Eggermann et al., 2003), Hcrt/Orx and MCH neurons were selected for whole-cell current clamp recording. Hcrt/Orx neurons were characterized by a low-threshold spike (LTS) followed by a slow afterdepolarization (ADP), indicative of a non-selective cation current (I_{CAN}), when depolarized from a hyperpolarized level, and the presence of membrane rectification indicative of an I_h current, when hyperpolarized from rest. Thirty cells having these characteristics were recorded in the perifornical region. Similar to the Nb-labeled Hcrt/Orx-IR cells described previously, they were characterized by a depolarized resting membrane potential (mean±S.E.M.=−46.2±0.51 mV, n=30). Seven of these cells were labeled with Nb and confirmed by double-labeling to contain Orx (Fig. 1C, E). Distinctly different from the Hcrt/Orx cells, MCH neurons were characterized by the lack of an LTS, ADP and I_h. Twenty cells with these characteristics were recorded in the region surrounding the fornix. Similar to the Nb-labeled MCH-IR cells described previously (Eggermann et al., 2003), they were also characterized by a relatively hyperpolarized resting membrane potential (mean±S.E.M.=−58.5±0.8 mV, n=20). Seven of these cells were labeled with Nb and confirmed by double-labeling to contain MCH (Fig. 1D, F).

NA was found to have opposite effects upon Hcrt/Orx and MCH neurons recorded in current clamp mode (Fig. 1G, H). NA depolarized the membrane and increased the spike frequency of the Hcrt/Orx neurons (at 10 μM, n=4/4 and at 100 μM, n=27/30; Fig. 1G). In contrast, NA hyperpolarized the membrane in MCH neurons (at 10 μM, n=4/4 and at 100 μM, n=15/20; Fig. 1H). The depolarizing action of NA on Hcrt/Orx neurons persisted after blocking Na^+-dependent action potentials with TTX (n=5/5) or all synaptic transmission with low Ca^{2+}/high Mg^{2+} (0.1 mM Ca^{2+}/10 mM Mg^{2+}, n=3/3). Similarly, the hyperpolarizing action of NA on MCH neurons persisted after blocking synaptic transmission with low Ca^{2+}/high Mg^{2+} (n=5/5), indicating that the observed effects were direct postsynaptic actions of NA on both cell types.

Carbachol also had opposite effects upon the two cell groups. It depolarized and excited Hcrt/Orx neurons (at 10 μM, n=18/18 and at 100 μM, n=26/26; Fig. 1I). This excitatory action was also present upon the Hcrt/Orx neurons in the presence of TTX (n=4/4) and low Ca^{2+}/high Mg^{2+} (n=5/5). In contrast, carbachol had a hyperpolarizing effect on the membrane potential of MCH neurons (at 10 μM, n=3/3 and at 100 μM, n=17/18; Fig. 1J). This