Electrophysiological study on the effects of leptin in rat dorsal motor nucleus of the vagus

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Leptin, a circulating protein of 16 kDa secreted primarily from white adipose tissue, is one of the key regulators of energy homeostasis (11, 37). It serves as a negative feedback signal of body adiposity through its actions in the central nervous system (CNS), particularly the hypothalamus, to regulate food intake and energy expenditure. At least 6 leptin receptors isoforms, Ob-Ra-f (23, 42, 43), have been identified, and the Ob-Rb isoform is believed to be pivotal in the regulation of energy homeostasis (11, 37). It serves as a negative feedback from white adipose tissue, is one of the key regulators of energy homeostasis (11, 37). It serves as a negative feedback from white adipose tissue, is one of the key regulators of energy homeostasis (11, 37). It serves as a negative feedback from white adipose tissue, is one of the key regulators of energy homeostasis (11, 37).

METHODS

Animals. Pregnant Sprague-Dawley (SD) rats were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and delivered to the Animal Center of Taipei Medical University 5 days before the due date. The use of animals was approved by the Institutional Animal Care and Use Committee of the Taipei Medical University. Neonatal rats of either sex were used.

Retrograde labeling of parasympathetic preganglionic neurons. Parasympathetic preganglionic neurons in the DMNV were retrograde-labeled with a systemically applied retrograde tracer, Fluorogold (Fluochrome, Denver, CO), as described in our previous study (20). Briefly, neonatal SD rats received an intraperitoneal injection of Fluorogold of 10 μg in 0.1 ml saline 2 or 3 days before the rats were

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killed for the immunohistochemical and electrophysiological experiments.

**Double-immuno-staining of Ob-R and retrograde-Fluorogold in the DMNV.** Three days after Fluorogold was injected, 10-day-old neonatal SD rats were transcardially perfused with cold PBS of 0.1 M followed by 4% paraformaldehyde/PBS. The brain was removed, postfixed in the same fixative for 2 h, and then immersed in 30% sucrose/PBS for 3 days. The brain stem containing the DMNV was coronally sectioned into 30-μm sections with a cryostat (Cryotome SME, Shandon, Astmoo, UK). Free-floating brain stem sections were processed for Ob-R and Fluorogold immunostaining using the avidin-biotin enzyme complex method (21). Briefly, sections were blocked with 10% normal horse serum and then incubated with goat polyclonal anti-Ob-R IgG (1:100, cat. no. sc-1835; Santa Cruz Biotechnology) for 24 h at room temperature followed by 24 h at 4°C with gentle agitation. After several washes with PBS, the sections were incubated with biotinylated horse anti-goat IgG (1:100, Vector Laboratories, Burlingame, CA) for 2 h followed by incubation with the avidin-biotinylated-alkaline phosphatase complex (1:100, Vector Laboratories) for 1 h at room temperature. After washing, sections were developed with a Vector Blue Kit (Vector Laboratories). The same sections were then processed for Fluorogold immunoreactivity with similar procedures of Ob-R immunostaining except that the following reagents differed: 1) 10% normal goat serum for blocking, 2) rabbit polyclonal Fluorogold antisera (1:3,000, Chemicon International, Temecula, CA) as the primary antibody, 3) biotinylated goat anti-rabbit IgG as the secondary antibody, 4) avidin-biotinylated peroxidase complex for signal amplification, and 5) Vector NovaRED kit for detection. The sections were examined with an upright microscope (Eclipse 80i, Nikon, Japan). The regions of interest were recognized by comparing section images with the atlases in *The Rat Brain in Stereotaxic Coordinates* (31). Cell counts were performed bilaterally using a ×20 objective at a 90-μm intervals (one-in-three series) from the level 400 μm caudal to the obex to the level 400 μm rostral to the obex. The ratio of Ob-R-labeled parasympathetic preganglionic DMNV neurons was calculated for each animal, and an averaged ratio was obtained from three neonatal rats. Immunohistochemical control experiments consisted of preadsorption of the antisera with an excess of antigen or omission of the primary antisera. Staining was absent in all of the immunohistochemical control experiments.

**Preparation of brain stem slices for electrophysiological experiments.** Brain stem slices containing the DMNV were prepared from neonatal SD rats of 8 to 14 days old. After anesthesia with ether, the brain stem was rapidly removed from the animal and placed in a chilled, oxygenated artificial cerebral spinal fluid (ACSF) containing the following compositions (in mM): 127 NaCl, 1.9 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgCl₂, 26 NaHCO₃, and 10 glucose. Three coronal sections of 400 μm, starting from about 600 μm caudal to the obex and moving rostrally, were sliced with a vibratome (Vibratome CA), low pass filter at 2 kHz, acquired using a personal computer and pClamp software (ver. 9.0, Axon Instruments) for later analysis. Membrane potentials reported have been corrected for a liquid-junction potential of −8 mV. The access resistance was less than 25 MΩ.

Whole cell patch-clamp recording was performed in both current-clamp and voltage-clamp modes. In the voltage-clamp experiments, the steady-state current-voltage (I-V) relationship was obtained before and during the application of leptin (PeproTech EC, London, UK) by applying a series of 1-s voltage command steps every 5 s from the holding potential of −60 mV to different potentials (~120 to 0 mV) at 10-mV increments in a TTX (0.5–1 μM)-containing ACSF (TTX was purchased from Sigma, St. Louis, MO). The current value was measured at the end of each command step. Currents elicited by such voltage commands in a control medium were subtracted from their counterparts in the presence of leptin to yield steady-state I–V curves of leptin-induced currents.

**Identification of parasympathetic preganglionic neurons in recorded neurons.** To identify whether the recorded neurons were parasympathetic preganglionic neurons, rats received an intraperitoneal injection of Fluorogold 2 to 3 days before the electrophysiological experiments were carried out (20). During the electrophysiological recordings, the patch electrodes were filled with a solution containing the fluorescent dye, Lucifer yellow (0.2%), which was allowed to diffuse into the recorded neuron, to identify the recorded neuron. At the end of the recordings, the slice was immersed in a solution of 4% paraformaldehyde/PBS overnight, and then transferred to a solution of 30% sucrose/PBS until further processing. The fixed slice was sectioned into 50-μm sections with a cryostat. Sections were viewed under an upright Nikon 80i epifluorescence microscope and the section containing Lucifer yellow-labeled neuron was selected for immunostaining of Fluorogold, as described previously (20). Briefly, the signal of the retrogradely Fluorogold was amplified by immunostaining with rabbit polyclonal Fluorogold antisera (Chemicon International, Temecula, CA) followed by detection with biotinylated goat anti-rabbit IgG and avidin-Texas red (Vector Laboratories). Fluorescent staining of the Lucifer yellow and Texas red was visualized using the fluorescent microscope.

**Data analysis.** Data are expressed as the means ± SE. Statistical analysis of the data was performed using Student’s t-test or one-factor ANOVA followed by multiple comparisons using the Student-Newman-Keuls test at P = 0.05.

**RESULTS**

Ob-R immunoreactivity in the DMNV of neonatal rats. Previous studies have demonstrated the existence of the Ob-R in the DMNV of adult rats (39). The use of neonatal rats in the present study was partly due to technical limitations of the blind patch-clamp recording in brain stem tissue. High plasma concentration of leptin is present in neonates of rodents and humans (1, 10, 15, 35), implying leptin as being a physiological regulator in newborn lives. To examine whether the Ob-R is present in parasympathetic preganglionic neurons of the DMNV of neonatal rats used in the present study, Fluorogold was administered systemically (ip) to labeled parasympathetic preganglionic neurons of the DMNV. Fluorogold is a retrograde tracer that can be taken up by nerve terminals but does not penetrate the blood-brain barrier. Therefore, systemic application of Fluorogold is a convenient method to label CNS neurons projecting to areas supplied by fenestrated capillaries or to the periphery (2, 24, 26). The distribution of the Ob-R in the DMNV neurons that project to the periphery was examined by the double immunostaining of Ob-R and Fluoro-gold. The result shows that most of Ob-R immunoreactive cells in the DMNV were Fluorogold-labeled, that is, parasympathetic

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preganglionic neurons. However, not all parasympathetic preganglionic neurons exhibited immunoreactivities to the Ob-R (Fig. 1). Ob-R immunoreactivity was detected in 45.6 ± 5.1% (n = 3) of the parasympathetic preganglionic neurons in the DMNV.

Passive membrane properties of DMNV neurons. Stable recordings were made from 80 DMNV neurons in brain stem slices harvested from 58 rats. The mean resting membrane potential, input resistance, and cell capacitance of the recorded DMNV neurons were −52 ± 1 mV, 366 ± 25 MΩ, and 31 ± 2 pF.

Effects of leptin on DMNV neurons. Under the current-clamp recording mode, leptin (30–300 nM) applied in the perfusion solution for 5 min caused membrane depolarization and hyperpolarization in 14 (17.5%) and 15 (17.6%) out of 80 recorded DMNV neurons, respectively (Fig. 2) and had no effect in the rest neurons. Both depolarization and hyperpolarization responses developed quickly and were often accompanied by changes in the activities of neuronal discharges. The passive membrane properties of the DMNV neurons with different responses to leptin are listed in Table 1, and no significant difference was found among the three groups. Three slices were obtained from each animal, and not more than one neuron was recorded in a slice. All responsive neurons of each experiment in the present study were from different animals.

Because the experiments were carried out in brain stem slices obtained from rats with ages ranging from 8 to 14 days old, the effect of animal age on differential responses of DMNV neurons to leptin was analyzed, and no significant effect was found among the three groups.

The concentration of leptin tested was from 10 to 300 nM. The lowest effective concentration of leptin was 30 nM. For a given concentration, the size of the response varied considerably among the different cells tested. However, a concentration-dependent manner of leptin-induced depolarization was observed for individual neurons. An example is shown in Fig. 2, A and B. The depolarization induced by leptin at 30–300 nM ranged from 4 to 16 mV, which could evoke firing activity in a silent neuron (Fig. 2, A and B). The hyperpolarization induced by leptin at 30–300 nM ranged from 4 to 28 mV, which could abolish the spontaneous neuronal activity (Fig. 2D). The duration of leptin-induced hyperpolarization was significantly longer than that of leptin-induced depolarization (Fig. 2). The duration of 90% recovery from leptin (300 nM)-induced responses was 18.3 ± 1.1 (n = 3) and 113.0 ± 22.1 (n = 3) min for depolarization and hyperpolarization, respectively. The long-lasting hyperpolarization response of leptin renders a repetitive application of leptin in a single neuron to evaluate the concentration-response relationship of leptin-induced hyperpolarization impracticable.

Under voltage-clamp recording mode, the leptin-induced depolarization and hyperpolarization were exhibited as an inward and outward currents, respectively, at a holding potential of −60 mV. TTX (0.5–1 μM), which eliminates action potentials by blocking voltage-gated sodium channels, was added to the ACSF to block any indirect effects due to the action of leptin on neighboring neurons in the brain stem slice. In the presence of TTX, leptin of 100 nM induced an inward current of 10 ± 3 pA in six tested DMNV neurons and an outward current of 19 ± 8 pA in four tested DMNV neurons. These results indicate that leptin affected neuronal activity of the DMNV via a direct action on DMNV neurons.

Ionic basis of leptin-induced depolarization and hyperpolarization. In this series of experiments, DMNV neurons were voltage-clamped to a holding potential of −60 mV. The steady-state I–V relationship was measured before (control curve) and during (leptin curve) the application of leptin (100 nM) by applying a series of command steps from the holding potential of −60 mV to different potentials (−120 to 0 mV) in a TTX-containing ACSF. The steady-state I–V curve of the leptin-induced inward current was obtained by subtracting the control curve from the leptin curve in the neurons, where leptin induced inward current (depolarized neurons) and outward current (hyperpolarized neurons), respectively.

Depolarized neurons. A representative steady-state I–V curve of leptin-induced inward currents at 100 nM is shown in Fig. 3. The I–V curve is characterized with a negative slope conductance, indicating that leptin caused a decrease in membrane conductance. The average reversal potential of leptin-induced inward current was −90 ± 3 mV (n = 6), which is close to the calculated equilibrium potential of potassium ions, −94 mV, under our experimental conditions, suggesting that potassium is the main ion involved in the leptin-induced depolarization/inward current. In a high-potassium (10 mM) medium, the amplitude of the leptin-induced inward currents was reduced, and the reversal potential of the steady-state I–V
been a critical breakthrough toward fully understanding energy homeostasis. Effects of leptin in the hypothalamus to control energy balance and food intake have been greatly explored both in rodents and humans. However, the Ob-R is widely distributed in the CNS, and leptin may have functions on multiple target sites, including the DMNV. The DMNV contains parasympathetic preganglionic neurons that are the major source of vagal efferents to the visceral organs in the thorax and upper abdomen and are involved in the regulation of gastrointestinal, cardiovascular, and pancreatic secretions, and many other autonomic functions (7, 25). Ob-R immunoreactivity has been detected in choline acetyltransferase-containing neurons, that is, the parasympathetic preganglionic neurons, in the DMNV of adult rats (40). Hosoi et al. (17) demonstrated that systemically applied leptin is able to activate Ob-Rb, in the DMNV of adult mice by detecting two biochemical functional markers of Ob-Rb receptors, STAT3 activation and of SOCS3 expression. The finding of Hosoi’s suggests a functional role of leptin in the regulation of parasympathetic activity through acting on the Ob-R in the DMNV of adult rats. In the present study, we demonstrated that, in neonatal rats, the Ob-R is also located on parasympathetic preganglionic neurons in the DMNV, and leptin can directly act on these neurons to cause membrane depolarization and hyperpolarization and, in turn, to increase or decrease neuronal excitability in different subgroups of DMNV neurons, most of which are parasympathetic preganglionic neurons. To our best knowledge, this is the first attempt to identify the respective leptin-responsive subgroups of DMNV neurons.

**Table 1. Passive membrane properties of DMNV neuron subgroups with different responses to leptin**

<table>
<thead>
<tr>
<th>Neuronal Responses to Leptin</th>
<th>Resting Membrane Potential, mV</th>
<th>Input Resistance, MO</th>
<th>Capacitance, pF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depolarization</td>
<td>$-54 \pm 3$</td>
<td>335 $\pm 80$</td>
<td>31 $\pm 6$</td>
</tr>
<tr>
<td>Hyperpolarization</td>
<td>$-52 \pm 1$</td>
<td>395 $\pm 61$</td>
<td>33 $\pm 3$</td>
</tr>
<tr>
<td>No response</td>
<td>$-52 \pm 1$</td>
<td>367 $\pm 29$</td>
<td>30 $\pm 3$</td>
</tr>
</tbody>
</table>

Values are expressed as means $\pm$ SE.
report providing functional evidence supporting that leptin directly affects the neuronal activity of parasympathetic preganglionic neurons of the DMNV.

Several in vivo studies have been performed to investigate the functional roles of leptin in the DMNV. Leptin when applied locally into the fourth ventricle inhibited gastric emptying in rats (40) and prevented fasting-induced anestrus in hamsters (36). Microinjection of leptin into the dorsal vagal complex, which contains the DMNV, caused an inhibition of food intake in rats (13). Moreover, increased leptin expression in the dorsal vagal complex reduced body weight and adiposity (4). Therefore, multiple functional roles are implicated for the leptin-responsive DMNV neurons, including the regulation of gastric functions, endocrine system, and energy homeostasis. However, the vagal efferents from the DMNV reach most of the visceral organs and tissues in the thorax and abdomen. Diverse vagal functions of the DMNV have only been partly explored for leptin’s involvement. Further studies will be needed to fully verify all physiological functions in which the different subgroups of leptin-responsive DMNV neurons are involved. In addition, Peters and coworkers (32, 33) have found that leptin selectively excites nodose ganglion neurons innervating the stomach and duodenum. Therefore, both the vagal afferents and efferents are sites of leptin’s actions.

Browning and colleagues (5) have found differential electrophysiological properties among the neuronal groups that project to different parts of the gastrointestinal tract. For example, the input resistance of fundus-projecting neurons, being 400 ± 25.3 MΩ, was significantly higher than that of other stomach-projecting neurons and cecum-projecting neurons, which are in the range of 291 ± 23 and 302 ± 22 MΩ. The input resistance (366 ± 25 MΩ) of the DMNV neurons recorded in the present study, are comparable to that reported by Browning et al. (5). However, no significant difference was found in the passive membrane properties among the leptin-depolarized, leptin-hyperpolarized and leptin-nonresponsive DMNV neurons. Therefore, it is infeasible to correlate the DMNV neuron by its responses to leptin (depolarization, hyperpolarization, or no response) to the functional subgroups identified by Browning and coworkers. Functional diversity of leptin-responsive neurons might account for the insignificant difference among the groups.

The plasma leptin concentrations are high in the neonates of rodents (1, 10) and humans (15), compared with adult animals or with Tanner stages 1 and 2 children, implying significant roles of leptin in the neonatal period. Functional studies strengthen this hypothesis. For example, leptin administered systemically reduce body weight and fat deposition in newborn rats (22, 45). In addition, in C57BL/6J mice, leptin-induced enhancement of the metabolic rate was detected at 17 days
postnatal age, whereas the inhibition of food intake by leptin developed later, after 28 days old (28). Evidence also supports that leptin plays a critical role in sexual development of humans and rodents (3, 6, 8). Thus, leptin’s direct effects on neonatal parasympathetic preganglionic DMNV neurons found in the present study might contribute to the regulation of neonatal development.

The circulating leptin is reported in a range from several to 50 ng/ml in neonatal rodents (1, 10). The lowest effective concentration of leptin in the present study is 30 nM, which is about 480 ng/ml, and is considerably higher compared with the reported physiological concentration of leptin. However, successful recordings are usually made on neurons located deep inside the brain stem slices since the neurons on surface are commonly damaged during the slicing process. Leptin is a big molecule with a molecular weight of 16 kDa and might not easily penetrate into the brain stem slice during a brief perfusion. The concentration of leptin reaching the recorded neuron is hard to be estimated yet it should be much lower than that in the bath solution.

Approximately 80% of the DMNV neurons are believed to be parasympathetic preganglionic neurons, and the remaining 20% neurons are likely to be local circuit interneurons or centrally projecting neurons, as suggested by Loewy and Spyer (25). Our results demonstrated that 29% (27/91) of the leptin-sensitive neurons are not Fluorogold retrograde-labeled neurons. This result suggests that leptin also affects nonparasympathetic neurons in the DMNV. Furthermore, the ratio of Ob-R-labeled parasympathetic preganglionic neurons, 45.6 ± 5.1%, is a little higher than the ratio of leptin-responsive cells, 36% (29/80), which includes the responsive neurons of nonparasympathetic preganglionic neurons. Whether leptin is involved in other regulatory mechanisms without affecting resting membrane potential needs further verification.

Our results demonstrate that potassium conductance(s) is likely the main underlying ionic mechanism of leptin-induced depolarization and hyperpolarization in the DMNV. Leptin was reported to cause membrane depolarization by activating a nonspecific cation channel in neurons of hypothalamic paraventricular nucleus (34) and proopiomelanocortin neurons of the arcuate nucleus in rats (9). Herein, we report a different mechanism, a reduction of potassium conductance, by which leptin induces membrane depolarization in DMNV neurons. On the other hand, the mechanism of leptin-induced hyperpolarization is consistent with the findings of others that an increase of potassium conductance underlies the inhibitory effects of leptin in neurons of other central areas, which include the glucose-sensitive neurons in ventromedial and arcuate nuclei of the hypothalamus (41) and hippocampal neurons (38).

The depolarization induced by leptin faded out rapidly upon removal of leptin. In contrast, after the washout of leptin, the leptin-induced hyperpolarization lasted for more than an hour. The latter result suggests that the potassium channels involved in leptin-induced hyperpolarization are likely activated by intracellular signaling cascades. Ob-R is a member of the cytokine receptor superfamily (42). The major brain-intrinsic leptin receptor isoform, Ob-Rb, is reported to couple with JAK/STAT3 transductional pathway (12). Activating Ob-Rb results in JAK2 activation, which, in turn, leads to the activation of other downstream kinase cascades, including MAPK and phosphatidylinositol 3-kinase (PI3K). It has been reported that leptin induces a long-lasting or irreversible hyperpolarization by activating an ATP-sensitive potassium channel (K\textsubscript{ATP}) in neurons of the ventromedial and arcuate nuclei in rat hypothalamus (41). A further study, using isolated rat arcuate neurons and mouse hypothalamic cell line GT1–7, demonstrates that leptin activates K\textsubscript{ATP} via a signaling pathway of PI3K-dependent depolymerization of actin filaments (27). A similar mechanism, by which leptin activates K\textsubscript{ATP} by a PI3K-dependent cortical actin rearrangement, has been reported for rat CRI-G1 insulinoma cells (14). A more recent study of Ning and coworkers’ (29) verifies that leptin inhibits PTEN (phosphatase and tension homology deleted on chromosome ten) activity, which in conjunction with increased PtdIns(3,4,5)P\textsubscript{3} (phosphatidylinositol 3,4,5-trisphosphate, i.e., the main products of PI3K activation) levels results in actin depolymerization and K\textsubscript{ATP} activation. The mechanism of leptin signaling has also been explored in hippocampal neurons, in which leptin activates BK channels via PI 3K-dependent reorganization of actin filaments (30, 38). It remains to be elucidated the signaling cascades and the associated potassium channels underlying the long-lasting hyperpolarization induced by leptin in DMNV neurons.

In summary, the present study demonstrates that leptin acts directly on parasympathetic preganglionic neurons in the DMNV of neonatal rats. This effect of leptin might contribute to its regulation of the parasympathetic outflow from the DMNV in neonatal rats and, perhaps, in adult animals too. Therefore, parasympathetic activity may play an important role in the physiological aspects of leptin. In
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In the future studies, it will be crucial to clarify the physiological functions in which leptin-responsive parasympathetic neurons in the DMNV are involved, as well as to identify the underlying ion channels and intracellular signaling of leptin-induced responses.

GRANTS

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