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

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Li T-L, Chiou L-C, Lin YS, Hsieh J-R, Hwang L-L. Electrophysiological study on the effects of leptin in rat dorsal motor nucleus of the vagus. *Am J Physiol Regul Integr Comp Physiol* 292: 2136–2143, 2007. First published February 15, 2007; doi:10.1152/ajpregu.00563.2006.—Immunoreactivity of leptin receptor (Ob-R) has been detected in rat dorsal motor nucleus of the vagus (DMNV). Here, we confirmed the presence of Ob-R immunoreactivity on retrograde-labeled parasympathetic preganglionic neurons in the DMNV of neonatal rats. The present study investigated the effects of leptin on DMNV neurons, including parasympathetic preganglionic neurons, by using whole cell patch-clamp recording technique in brain stem slices of neonatal rats. Leptin (30–300 nM) induced membrane depolarization and hyperpolarization, respectively, in 14 and 15 out of 80 DMNV neurons tested. Both leptin-induced inward and outward currents persisted in the presence of TTX, indicating that leptin affected DNMV neurons postsynaptically. The current-voltage (I–V) curve of leptin-induced inward currents is characterized by negative slope conductance and has an average reversal potential of -90 ± 3 mV. The reversal potential of the leptin-induced inward current was shifted to a more positive potential level in a high-potassium medium. These results indicate that a decrease in potassium conductance is likely the main ionic mechanism underlying the leptin-induced depolarization. On the other hand, the I–V curve of leptin-induced outward currents is characterized by positive slope conductance and has an average reversal potential of -88 ± 3 mV, suggesting that an increase in potassium conductance may underlie leptin-induced hyperpolarization. Most of the leptin-responsive DMNV neurons were identified as being parasympathetic preganglionic neurons. These results suggest that the DMNV is one of the central target sites of leptin, and leptin can regulate parasympathetic outflow from the DMNV by directly acting on the parasympathetic preganglionic neurons of the DMNV.

Ob-R; brain slice; potassium channel; whole cell patch-clamp; immunohistochemistry

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-$R_{a\text{-}f}$ (23, 42, 43), have been identified, and the Ob-R_b isoform is believed to be pivotal in the regulation of energy homeostasis. Intense Ob-R immunoreactivity was detected not only in the hypothalamus but also in other brain regions, including many nuclei in the brain stem (39). Hosoi and coworkers (17) demonstrated that leptin intravenously applied activated Ob-R_b in the nucleus tractus solitarii, lateral parabrachial nucleus, central gray and dorsal motor nucleus of the vagus (DMNV) of the brain stem, by detecting two biochemical markers of the activation of Ob-R_b, that is, the activation of signal transducers and activators of transcription (STAT3) and the expression of suppressor of cytokine signaling 3 (SOCS3) (17). They, therefore, proposed that the brain stem is a direct target site of leptin in the brain. Several lines of evidence are in agreement with this hypothesis. First, when injected into the fourth ventricle, leptin was found to inhibit gastric emptying in rats (40) and to reverse fasting-induced anestrus in hamsters (36). Further, Grill and colleagues (13) showed that leptin microinjected into the dorsal vagal complex suppressed food intake in rats. A recent report of Williams and Smith (44) demonstrates that leptin directly hyperpolarized NTS neurons, providing direct functional evidence that supports the hypothesis proposed by Hosoi et al. (17).

Several electrophysiological studies have been conducted in the hypothalamus, a primary site of leptin's action to influence energy homeostasis, and demonstrate that leptin acts in several hypothalamic areas by changing neuronal excitabilities (9, 16, 34, 41). However, little electrophysiological study was carried out in the brain stem nuclei, which are considered to be potential action targets of leptin. The DMNV is one of the brain stem nuclei that exhibit Ob-R immunoreactivity (39) and a crucial area involved in the regulation of feeding. It is where the parasympathetic preganglionic neurons are located and provides the major source of parasympathetic efferents to subdiaphragmatic targets (25). The present study, therefore, investigated how leptin affects the neuronal activities of DMNV neurons, including the retrograde-labeled parasympathetic preganglionic neurons, using whole cell patch-clamp recording technique in brain stem slices.

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-immunostaining 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, Astmoor, 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 antiserum with an excess of antigen or omission of the primary antiserum. 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 anesthetization 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 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 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 1000 classic; St. Louis, MO). The brain stem slices were then incubated in an oxygenated ACSF at room temperature for at least 1 h before the start of the recording.

Electrophysiological recording. After a 1-h recovery in oxygenated ACSF, one slice was transferred to a recording chamber and continuously perfused with an oxygenated ACSF at a rate of 1 to 3 ml/min throughout the entire period of the experiment. All reagents were applied in the perfused ACSF. A high-potassium ACSF was prepared by substituting NaCl with KCl for some experiments. All experiments were conducted at room temperature (21 ± 1°C).

The whole cell patch-clamp recording technique employed was similar to that described previously (18–20). Glass microelectrodes filled with an electrode solution had a resistance of 2 to 5 MΩ. The electrode solution contained (in mM) 125 K^+ gluconate, 5 KCl, 1 $MgCl_2$, 0.4 $CaCl_2$, 5 ATP, 0.3 GTP, 2 EGTA, 10 HEPES, 10 sucrose, and in some cases, 0.2% Lucifer yellow, with a pH of 7.2. Signals were recorded with an Axopatch-1D (Axon Instruments, Foster City, 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 retrograded Fluorogold was amplified by immunostaining with rabbit polyclonal Fluorogold antisera (Chemicon International, Temecula, CA) followed by detection with biotinylated goat anti-rabbit IgG and then 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

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.

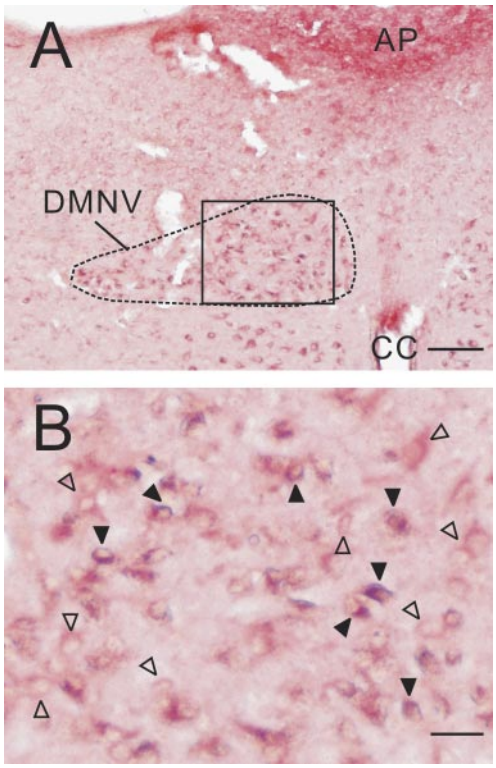

Fig. 1. Presence of Ob-R-immunoreactivity on Fluorogold-labeled parasympathetic preganglionic neurons in the dorsal motor nucleus of the vagus (DMNV). *A*: A photomicrograph of a brain stem section containing the DMNV neurons double-stained with Fluorogold- and Ob-R-immunoreactivities in red and blue, respectively. *B*: A higher magnification of the rectangle area outlined in *A*. Solid arrowheads point to representative double-stained neurons, and open arrowheads indicate neurons stained only with Fluorogold immunoreactivities. AP, area postrema; CC, central canal; DMNV, dorsal motor nucleus of the vagus nerve. Scale bar: 100 μm in *A*; 25 μm in *B*.

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. 2*D*). 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

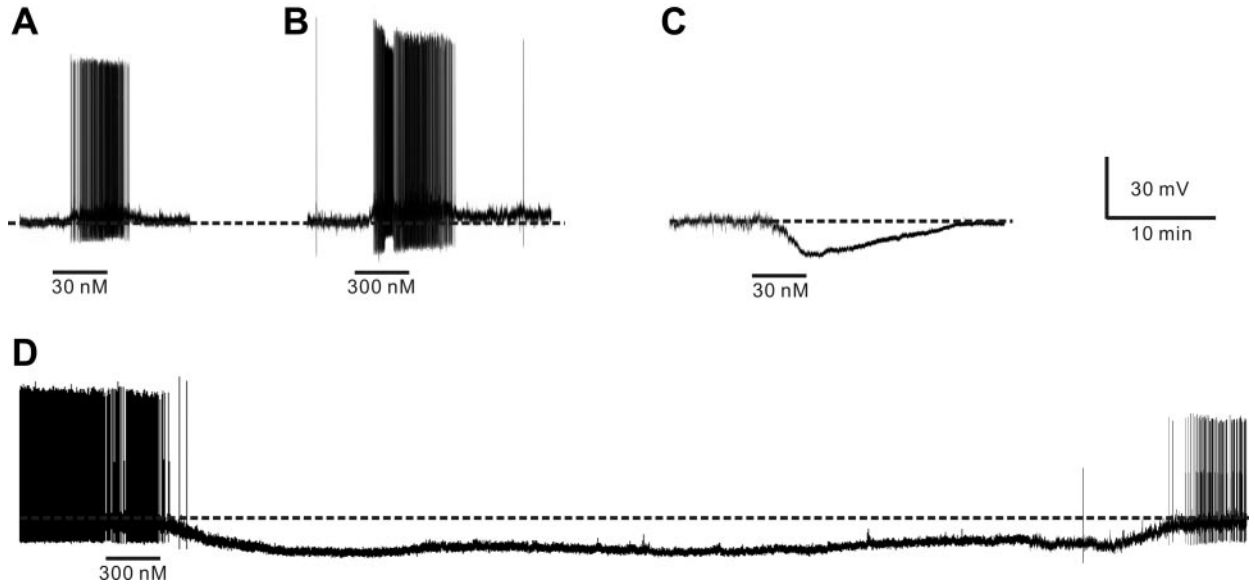

Fig. 2. Leptin-induced depolarization and hyperpolarization in DMNV neurons. *A* and *B*: leptin of 30 and 300 nM applied in a bath solution caused membrane depolarization accompanied by intensified spontaneous discharges in a DMNV neuron. The resting potential of this neuron was −55 mV. *C* and *D*: leptin of 30 and 300 nM applied in bath solution caused membrane hyperpolarization associated with cessation of the spontaneous discharges in 2 DMNV neurons. The resting levels of neuron in *C* and *D* were −53 and −45 mV, respectively.

curve was shifted to −57 ± 7 mV (n = 3), a more-positive potential level, as predicted by the Nernst equation for a potassium-selective conductance. Therefore, a decrease in potassium conductance is likely the main ionic mechanism underlying leptin-induced depolarization/inward current. Significant outward rectification, which is manifested by increased slope conductance in the direction of membrane depolarization, was noticed in the I–V relationship of leptin-induced inward current (Fig. 3*B*).

Hyperpolarized neurons. Under voltage-clamp recording, leptin-induced hyperpolarization was demonstrated to be an outward current. The steady-state I–V curve of the leptin (100 nM)-induced outward currents is characterized with a positive slope conductance and has a reversal potential of around −90 mV (Fig. 4). The average reversal potential of the leptin-induced outward current was −88 ± 3 mV (n = 4). The results indicated that an increase of potassium conductance might underlie leptin-induced hyperpolarization. No evident inward or outward rectification was observed in the I–V relationship of leptin-induced outward current (Fig. 4*B*).

Most of leptin-responsive neurons are parasympathetic preganglionic neurons. The DMNV contains parasympathetic preganglionic neurons that send projection to the vagal ganglia and are the major source of vagal efferents to the visceral organs in the thorax and upper abdomen (25). Parasympathetic preganglionic neurons in the DMNV were identified in leptin-responsive neurons with a positive immunostaining of Fluorogold in the cytosol. Among 7 leptin-responsive DMNV neurons, five neurons, including three leptin-depolarized and two leptin-hyperpolarized neurons, were identified as parasympathetic preganglionic neurons (Fig. 5). The other two nonparasympathetic preganglionic neurons were leptin-depolarized.

DISCUSSION

The discovery of leptin, a product of the ob gene (46), has 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-R_b in the DMNV of adult mice by detecting two biochemical functional markers of Ob-R_b 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

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

Neuronal Responses to Leptin	Resting Membrane Potential, mV	Input Resistance, MΩ	Capacitance, pF
Depolarization	−54±3	335±80	31±6
Hyperpolarization	−52±1	395±61	33±3
No response	−52±1	367±29	30±3

Values are expressed as means ± SE.

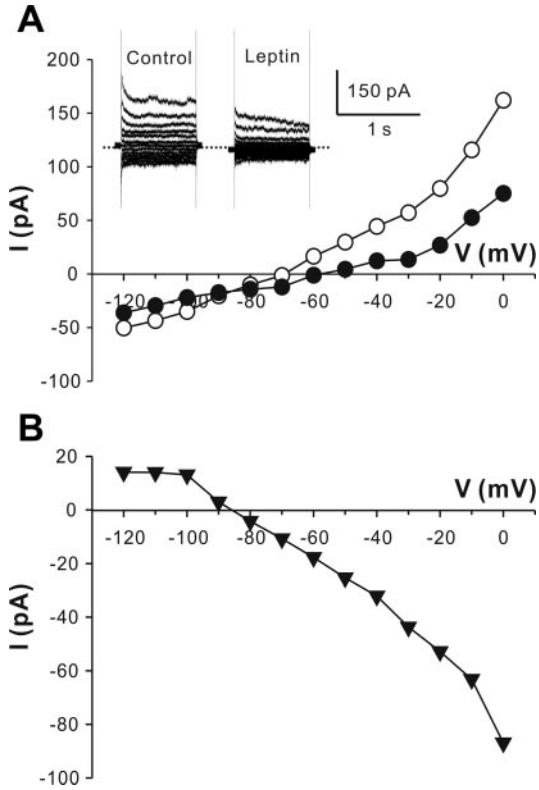

Fig. 3. Steady-state I–V relationship of leptin-induced inward currents in a DMNV neuron. The insets in *A* are original current traces in response to a series of command steps (from −60 mV to −120 to 0 mV) recorded before and during the application of leptin (100 nM). *A*: I–V curves measured before (open circles) and during (solid circles) the application of leptin (100 nM). Leptin decreased the neuronal input resistance, as evidenced by a decreased slope of the I–V curve. *B*: I–V relationship of leptin-induced currents (solid triangles) with a reversal potential close to −90 mV. The experiments were performed in the presence of TTX.

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 inervating 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, hyperpoloarization, 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

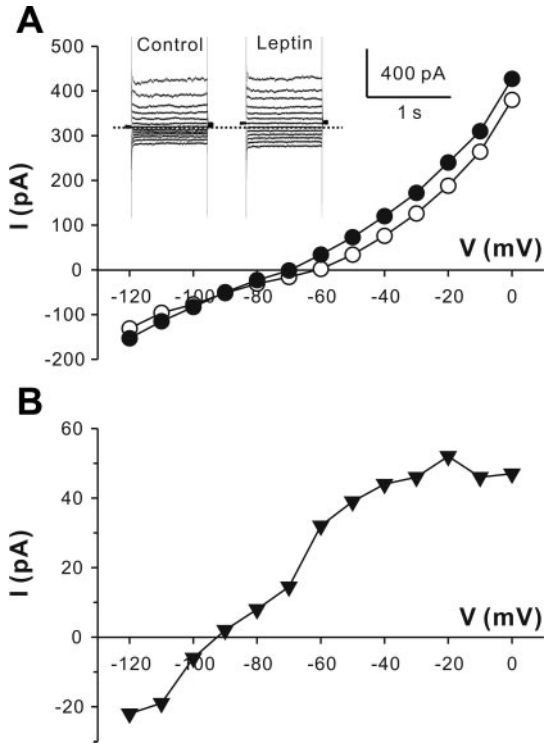

Fig. 4. Steady-state I–V relationship of leptin-induced outward currents in a DMNV neuron. The insets in *A* are original current traces in response to a series of command steps (from −60 mV to −120 to 0 mV) recorded before and during the application of leptin (100 nM). *A*: I–V curves measured before (open circles) and during (solid circles) the application of leptin (100 nM). Leptin increased the neuronal input resistance, as evidenced by an increased slope of the I–V curve. *B*: I–V relationship of leptin-induced currents (solid triangles) with a reversal potential close to −90 mV. The experiments were performed in the presence of TTX.

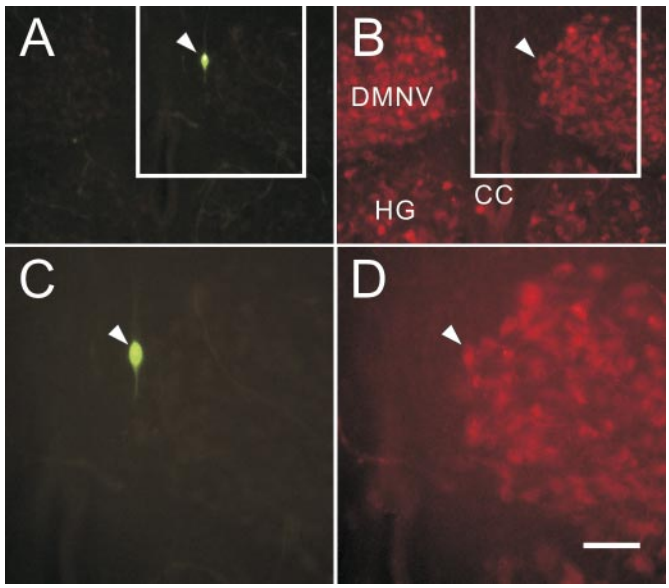

Fig. 5. A leptin-responsive neuron identified as a parasympathetic preganglionic neuron in the DMNV. The rat from which this brain stem section was taken had received an injection of a retrograde tracer, Fluorogold, 3 days before the recordings were made. *A*: micrograph of a leptin-responsive neuron intracellularly labeled with Lucifer yellow (green-yellow color). *B*: Fluorogold-filled parasympathetic preganglionic neurons in the DMNV (red color) of the same area in the brain stem slice shown in *A*, demonstrating that the leptin-responsive neuron is a parasympathetic preganglionic neuron in DMNV. *C* and *D*: higher magnification of the rectangle areas outlined in *A* and *B*, respectively. DMNV, dorsal motor nucleus of the vagus nerve; HG, hypoglossal nucleus; CC, central canal. Scale bar: 100 μm in *A* and *B*; 50 μm in *C* and *D*.

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 considerablely 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 brainbstem slice during a brief perfusion of leptin. 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% (2/7) 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-R_b, is reported to couple with JAK/STAT3 transductional pathway (12). Activating Ob-R_b 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_{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_{ATP} via a signaling pathway of PI3K-dependent depolymerization of actin filaments (27). A similar mechanism, by which leptin activates K_{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_3 (phosphatidylinositol 3,4,5-trisphosphate, i.e., the main products of PI3K activation) levels results in actin depolymerization and K_{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

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.

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REFERENCES

1. **Ahima RS, Prabakaran D, Flier JS.** Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. *J Clin Invest* 101: 1020–1027, 1998.
2. **Ambalavanar R, Morris R.** Fluoro-gold injected either subcutaneously or intravascularly results in extensive retrograde labeling of CNS neurones having axons terminating outside the blood-brain barrier. *Brain Res* 505: 171–175, 1989.
3. **Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK, Steiner RA.** Leptin is a metabolic signal to the reproductive system. *Endocrinology* 137: 3144–3147, 1996.
4. **Boghossian S, Lecklin A, Dube MG, Kalra PS, Kalra SP.** Increased leptin expression in the dorsal vagal complex suppresses adiposity without affecting energy intake and metabolic hormones. *Obesity* 14: 1003–1009, 2006.
5. **Browning KN, Renehan WE, Travagli RA.** Electrophysiological and morphological heterogeneity of rat dorsal vagal neurones which project to specific areas of the gastrointestinal tract. *J Physiol* 517: 521–532, 1999.
6. **Chehab FF, Lim ME, Lu R.** Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 12: 318–320, 1996.
7. **Cheng SB, Lu GQ.** Progress in the study of the dorsal motor nucleus of the vagus nerve. *Prog Physiol Sci* 27: 13–18, 1996.
8. **Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B.** A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 392: 398–401, 1998.
9. **Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ.** Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411: 480–484, 2001.
10. **Devaskar SU, Ollesch C, Rajakumar RA, Rajakumar PA.** Developmental changes in ob gene expression and circulating leptin peptide concentrations. *Biochem Biophys Res Commun* 238: 44–47, 1997.
11. **Friedman JM, Halaas JL.** Leptin and the regulation of body weight in mammals. *Nature* 395: 763–770, 1998.
12. **Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC.** Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci USA* 93: 6231–6235, 1996.
13. **Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG.** Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143: 239–246, 2002.
14. **Harvey J, Hardy SC, Irving AJ, Ashford ML.** Leptin activation of ATP-sensitive K^+ (KATP) channels in rat CRI-G1 insulinoma cells involves disruption of the actin cytoskeleton. *J Physiol* 52: 95–107, 2000.
15. **Hassink SG, de Lancey E, Sheslow DV, Smith-Kirwin SM, O'Connor DM, Considine RV, Opentanova I, Dostal K, Spear ML, Leef K, Ash M, Spitzer AR, Funanage VL.** Placental leptin: an important new growth factor in intrauterine and neonatal development? [Online]. *Pediatrics* 100: E1, 1997.
16. **Honda K, Narita K, Murata T, Higuchi T.** Leptin affects the electrical activity of neurones in the hypothalamic supraoptic nucleus. *Brain Res Bull* 57: 721–725, 2002.
17. **Hosoi T, Kawagishi T, Okuma Y, Tanaka J, Nomura Y.** Brain stem is a direct target for leptin's action in the central nervous system. *Endocrinology* 143: 3498–3504, 2002.
18. **Hwang LL, Dun NJ.** 5-Hydroxytryptamine responses in immature rat rostral ventrolateral medulla neurons in vitro. *J Neurophysiol* 80: 1033–1041, 1998.
19. **Hwang LL, Dun NJ.** 5-Hydroxytryptamine modulates multiple conductances in immature rat rostral ventrolateral medulla neurones in vitro. *J Physiol* 517: 217–228, 1999.
20. **Hwang LL, Chen CT, Dun NJ.** Mechanisms of orexin-induced depolarizations in rat dorsal motor nucleus of vagus neurones in vitro. *J Physiol* 537: 511–520, 2001.
21. **Hwang LL, Chen CT, Li TL, Chiu CZ, Chi SF.** Central pressor effects of CART peptides in anesthetized rats. *Neuropeptides* 38: 69–76, 2004.
22. **Kraeft S, Schwarzer K, Eiden S, Nuesslein-Hildesheim B, Preibisch G, Schmidt I.** Leptin responsiveness and gene dosage for leptin receptor mutation (fa) in newborn rats. *Am J Physiol Endocrinol Metab* 276: E836–E842, 1999.
23. **Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM.** Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379: 632–635, 1996.
24. **Leong SK, Ling EA.** Labelling neurons with fluorescent dyes administered via intravenous, subcutaneous or intraperitoneal route. *J Neurosci Methods* 32: 15–23, 1990.
25. **Loewy AD, Spyer KM.** Vagal preganglionic neurons. In: *Central Regulation of Autonomic Functions*, edited by Loewy AD and Spyer KM. New York, Oxford University Press, 1990.
26. **Merchenthaler I.** Neurons with access to the general circulation in the central nervous system of the rat: a retrograde tracing study with Fluoro-gold. *Neuroscience* 44: 655–662, 1991.
27. **Mirshamsi S, Laidlaw HA, Ning K, Anderson E, Burgess LA, Gray A, Sutherland C, Ashford ML.** Leptin and insulin stimulation of signaling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation [Online]. *BMC Neurosci* 5: 54, 2004.
28. **Mistry AM, Swick A, Romsos DR.** Leptin alters metabolic rates before acquisition of its anorectic effect in developing neonatal mice. *Am J Physiol Regul Integr Comp Physiol* 277: R742–R747, 1999.
29. **Ning K, Miller LC, Laidlaw HA, Burgess LA, Perera NM, Downes CP, Leslie NR, Ashford ML.** A novel leptin signaling pathway via PTEN inhibition in hypothalamic cell lines and pancreatic beta-cells. *EMBO J* 25: 2377–2387, 2006.
30. **O'Malley D, Irving AJ, Harvey J.** Leptin-induced dynamic alterations in the actin cytoskeleton mediate the activation and synaptic clustering of BK channels. *FASEB J* 19: 1917–1919, 2005.
31. **Paxinos G, Watson C.** *The Rat Brain in Stereotaxis Coordinates* (3rd ed.). San Diego, CA: Academic Press, 1997.
32. **Peters JH, Ritter RC, Simasko SM.** Leptin and CCK modulate complementary background conductances to depolarize cultured nodose neurons. *Am J Physiol Cell Physiol* 290: C427–C432, 2006.
33. **Peters JH, Ritter RC, Simasko SM.** Leptin and CCK selectively activate vagal afferent neurons innervating the stomach and duodenum. *Am J Physiol Regul Integr Comp Physiol* 290: R1544–R1549, 2006.
34. **Powis JE, Bains JS, Ferguson AV.** Leptin depolarizes rat hypothalamic paraventricular nucleus neurons. *Am J Physiol Regul Integr Comp Physiol* 274: R1468–R1472, 1998.
35. **Rayner DV, Dalgliesh GD, Duncan JS, Hardie LJ, Hoggard N, Trayhurn P.** Postnatal development of the ob gene system: elevated leptin levels in suckling *falfa* rats. *Am J Physiol Regul Integr Comp Physiol* 273: R446–R450, 1997.
36. **Schneider JE, Goldman MD, Tang S, Bean B, Ji H, Friedman MI.** Leptin indirectly affects estrous cycles by increasing metabolic fuel oxidation. *Horm Behav* 33: 217–228, 1998.
37. **Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG.** Central nervous system control of food intake. *Nature* 404: 661–671, 2000.
38. **Shanley LJ, O'Malley D, Irving AJ, Ashford ML, Harvey J.** Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. *J Physiol* 545: 933–944, 2002.
39. **Shioda S, Funahashi H, Nakajo S, Yada T, Maruta O, Nakai Y.** Immunohistochemical localization of leptin receptor in the rat brain. *Neurosci Lett* 243: 41–44, 1998.
40. **Smedh U, Hakansson ML, Meister B, Uvnas-Moberg, K.** Leptin injected into the fourth ventricle inhibits gastric emptying. *Neuroreport* 9: 297–301, 1998.
41. **Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford ML.** Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature* 390: 521–525, 1997.
42. **Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI.** Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83: 1263–1271, 1995.

43. **Wang MY, Zhou YT, Newgard CB, Unger RH.** A novel leptin receptor isoform in rat. *FEBS Lett* 392: 87–90, 1996.
44. **Williams KW, Smith BN.** Rapid inhibition of neural excitability in the nucleus tractus solitarii by leptin: implications for ingestive behaviour. *J Physiol* 573: 395–412, 2006.
45. **Yuan CS, Attele AS, Zhang L, Lynch JP, Xie JT, Shi ZQ.** Leptin reduces body weight gain in neonatal rats. *Pediatr Res* 48: 380–383, 2000.
46. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM.** Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425–432, 1994.