0026-895X/96/020230-06$3.00/0

MOLECULAR PHARMACOLOGY, 50:230–235 (1996).

ACCELERATED COMMUNICATION

Leptin, the *Obese* Gene Product, Rapidly Modulates Synaptic Transmission in the Hypothalamus

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Received March 19, 1996; Accepted April 23, 1996

SUMMARY

We examined the effects of leptin, the product of the *obese* gene, on synaptic transmission in the arcuate nucleus in rat hypothalamic slices. Both leptin and neuropeptide Y (NPY) reduced the evoked glutamatergic excitatory postsynaptic current in the arcuate nucleus. NPY also depressed the GABAergic inhibitory postsynaptic current, although leptin had no effect. Leptin also decreased the input resistance of arcuate neurons, and this was accompanied by the activation of an outward current at depolarized potentials. Leptin modulated Ca^{2+} signals in acutely isolated arcuate neurons. In some cells, the intracellular calcium concentration rise produced by 50 mM K^+ was decreased, whereas in others it was increased. However, leptin produced no effects on synaptic transmission and little or no effect on Ca^{2+} signaling in the hypothalamus of Zucker fatty rats that contain mutated leptin receptors. On the other hand, NPY exhibited synaptic modulatory effects in Zucker lean and fatty rats. These data suggest that leptin can produce rapid synaptic modulatory effects in the arcuate nucleus, which may contribute to its effects on food intake.

Leptin, the product of the *ob* gene, is a 146-amino acid protein secreted by adipocytes (1) that reduces appetite and increases energy expenditure (2–6). *ob/ob* mice, which do not produce leptin, are massively obese, and the injection of recombinant leptin causes these animals to lose weight (2–6). In contrast, the injection of NPY into the hypothalamus stimulates appetite and food intake (7–9), suggesting that leptin and NPY may function together to regulate feeding and body weight (5). The identification of high affinity binding sites for ^{125}I-labeled recombinant mouse leptin and of leptin receptors in the rat hypothalamus is consistent with this hypothesis (5, 10–12). Furthermore, leptin reduces NPY mRNA and NPY release from the ARC of *ob/ob* mice (5). In the current study, we demonstrate that leptin produces rapid synaptic modulatory effects in the ARC of normal but not obese Zucker (*fa/fa*) rats, which express a truncated receptor for leptin (11, 13–16). Thus, one of the normal functions of leptin is to inhibit the activity of neurons in the ARC that regulate feeding.

Materials and Methods

Electrophysiology. Coronal slices (160–175 μm for electrophysiology, 400 μm for dissociation) were prepared from 16–50-day-old Sprague-Dawley (12 animals), obese Zucker (*fa/fa*) (three animals), or lean Zucker (*Fa/?*) (three animals) rats as described previously (17). ARC neurons were identified visually with the use of a microscope at 400× magnification. Voltage-clamp recordings (V_{hold}, −60 to −70 mV) were made in the discontinuous mode of the amplifier (7–14-kHz switching rate), which allowed for complete settling of the

This work was supported by United States Public Health Service Grants DA02121, DA02575, NS33502, and MH40165 (R.J.M.); Pharmacology Training Grant 5T32-GM07151-21 (C.C.L.); and United States Public Health Service Grants DK20595 and DK44840 and an unrestricted grant for cardiovascular research from Bristol-Myers Squibb and the Howard Hughes Medical Institute (G.I.B.).

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ABBREVIATIONS: NPY, neuropeptide Y; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; VMH, ventromedial nucleus of the hypothalamus; ARC, arcuate nucleus of the hypothalamus; DNQX, 2,3-dihydroxy-6,7-dinitroquinoxaline; $[Ca^{2+}]_i$, intracellular calcium concentration; aCSF, artificial cerebrospinal fluid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PBS, phosphate-buffered saline; PVN, paraventricular nucleus; JAK/STAT, janus kinase/signal transducers and activators of transcription.

headstage signal (18). Series resistance was <5 MΩ. Patch electrodes were filled with K^+-gluconate (for EPSC recordings) containing 145 mM K^+-gluconate, 2 mM $MgCl_2$, 5 mM HEPES, 1.1 mM EGTA, 0.1 mM $CaCl_2$, and 5 mM K_2ATP (pH 7.2 with KOH) or CsCl (for IPSC recordings) containing 145 mM CsCl, 2 mM $MgCl_2$, 5 mM HEPES, 1.1 mM EGTA, 0.1 mM $CaCl_2$, and 5 mM Mg_2ATP (pH 7.2 with CsOH). Slices were continuously bathed with aCSF (containing 126 mM NaCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3 mM KCl, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose; Ref. 18). Perforated-patch recordings were made with amphotericin B (20 μg/ml) containing electrodes. Recombinant mouse leptin was a gift from Amgen, Inc. (Thousand Oaks, CA) and was prepared as a 1.28 mg/ml stock solution in PBS. It was further diluted in aCSF containing 0.2% ovalbumin as a carrier (18). NPY was a gift from L. Grundemar (University of Lund, Sweden) and was prepared as a 10^{-3} M stock solution in water. All drugs were diluted in aCSF at the appropriate concentrations and were delivered in the perfusate. DNQX (10 μM) or bicuculline (10 μM) was present throughout experiments concerning IPSCs or EPSCs, respectively. Synaptic stimulation was delivered at 0.1 Hz using a bipolar stimulating electrode (300 μsec, 3–18 V). Data points illustrate consecutive 1–2-min running average values for peak currents. Individual traces are the averages of three consecutive evoked synaptic events. Current-voltage plots (Fig. 1) were derived from the mean of three 300-msec (whole-cell recording) or 3-sec (perforated-patch recording) voltage steps from V_{hold} of −70 mV. Input resistance for each cell studied with K^+-gluconate electrodes (15 cells) was determined by 1-sec, 20-pA current injections under current clamp conditions from cell V_m of −50 to −65 mV. Individual and population statistical analyses was performed with analysis of variance for repeated measures followed by Student-Newman-Keuls posthoc comparisons as described previously (18).

$[Ca^{2+}]_i$ imaging microfluorimetry. $[Ca^{2+}]_i$ elevations after 2.5-sec fast applications of saline containing 50 mM KCl (with 0.1 mg/ml ovalbumin) were measured using ratiometric imaging microfluorimetry of Fura-2/AM-loaded cells as described previously (19). The ARC nucleus was micropunched out of hypothalamic slices, and dissociated cells were prepared in a manner similar to that described by Kaneda *et al.* (20). Leptin was bath-applied 60 sec before applications of 50 K^+ containing leptin. The peaks in $[Ca^{2+}]_i$ were analyzed graphically and by a custom program written for MATLAB (The Math Works, Natick, MA). The program calculated peak height, 10–90% fall time, area, and width at 25% of peak height. Positive leptin responses were scored by consistent changes in basal $[Ca^{2+}]_i$ or treatment-related changes in peak height, width, or fall time trends. In some experiments, the mean responses before, during, and after washout of leptin were compared with analysis of variance and Kolmogorov-Smirnov comparisons.

Staining. NPY immunoreactivity were identified from representative hypothalamic slices (Fig. 2B) and isolated cells used for Ca^{2+} measurements (Fig. 3). Rats were anesthetized with Metofane (Pitman-Moore, Mundeline, IL) and killed by transcardiac perfusion with PBS followed by 4% paraformaldehyde. Whole brains were removed and stored in 4% paraformaldehyde with 30% sucrose at 4°. Sections (20 μm) were prepared using a cryostat, permeabilized using 0.25% Triton X-100 in PBS for 30 min, rinsed in PBS containing 0.1% Tween 20, and then rinsed in blocking solution (PBS with 0.1% Tween 20 and 2% bovine serum albumin) at 4° for overnight. The sections were treated with a 1:200 dilution of rabbit anti-NPY polyclonal antibody (lot 76195199; Chemicon International, Temecula, CA) in blocking solution for 3 hr, rinsed three times with PBS containing Tween 20 (0.1%), and then treated with a 1:40 dilution of Cy3-conjugated Affinipure anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) for 2 hr. After three rinses with PBS/Tween 20, the sections were treated with 2% 1,4-diazabicyclo-[2,2,2]octane (Sigma Chemical Co., St. Louis, MO) to prevent fading and mounted onto 1% gelatin-coated slides. The sections were visualized using excitation at 510–560 nm and a 590-nm barrier filter. Images were captured on Ektachrome film or digitally using Image 1 software (Universal Imaging, West Chester, PA). Control sections were treated as above but without the primary antibody.

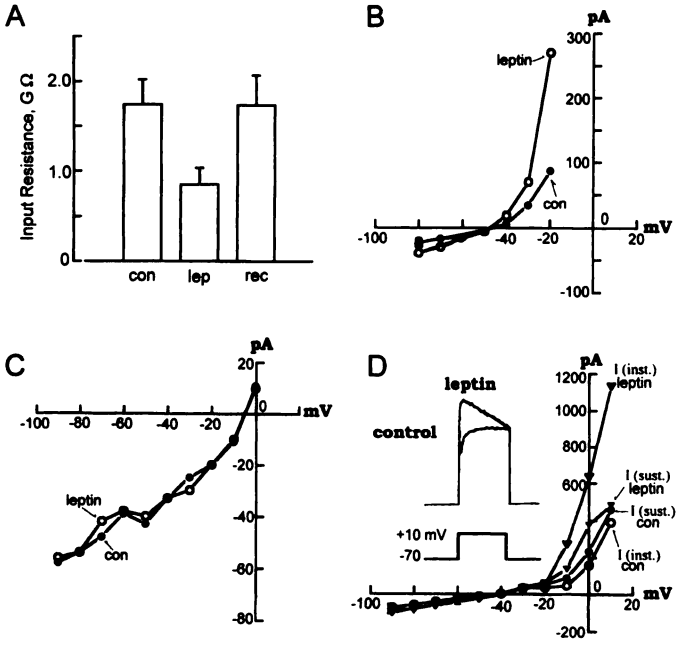

Fig. 1. A, Leptin reduced cell input resistance in ARC neurons (15 experiments). Whole-cell current-voltage plots from neurons recorded with K^+-gluconate (B) or CsCl-filled (C) electrodes. ●, Control (*con*) current; ○, current in presence of 100 ng/ml leptin. D, Instantaneous [*I (inst.)*] and sustained [*I (sust.)*] current-voltage plot from a perforated-patch recording of an ARC neuron in the absence or presence of leptin (100 ng/ml). *Inset*, Representative current response of this neuron to a 3-sec voltage step in the absence or presence of leptin.

Results and Discussion

Whole-cell and perforated-patch recordings were made from hypothalamic neurons in coronal brain slices from 16–50-day-old Sprague-Dawley and lean (*Fa/?*) or obese (*fa/fa*) Zucker rats (Fig. 2A). The ARC possesses a high density of NPY-containing neurons which project to the PVN as well as sending recurrent collaterals to other ARC neurons (21). Neurons in the region of the ARC from which we recorded contained many of these NPY-positive cells (Fig. 2B). Recordings were also made from the VMH, of which lesions lead to uncontrolled feeding (22). Stimulation using electrodes placed ventrolateral to these areas evoked excitatory/inhibitory postsynaptic potential complexes recorded under current clamp conditions with K^+ gluconate electrodes. Under these conditions, excitatory postsynaptic potentials produced depolarization and inhibitory postsynaptic potentials produced hyperpolarization from the resting potential (−50 to −65 mV). These components of synaptic transmission were studied under voltage-clamp conditions and separated pharmacologically by the γ-aminobutyric $acid_A$ antagonist bicuculline for examining EPSCs and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid antagonist DNQX for examining IPSCs (Fig. 2, C and D).

Perfusion of slices with leptin (100 ng/ml) produced a robust inhibition of the EPSC recorded in the ARC but failed to inhibit the IPSC (Fig. 2, C–E). This effect of leptin was rapid (<1 min) and fully reversible (4–9 min) in 11 of 15 record-

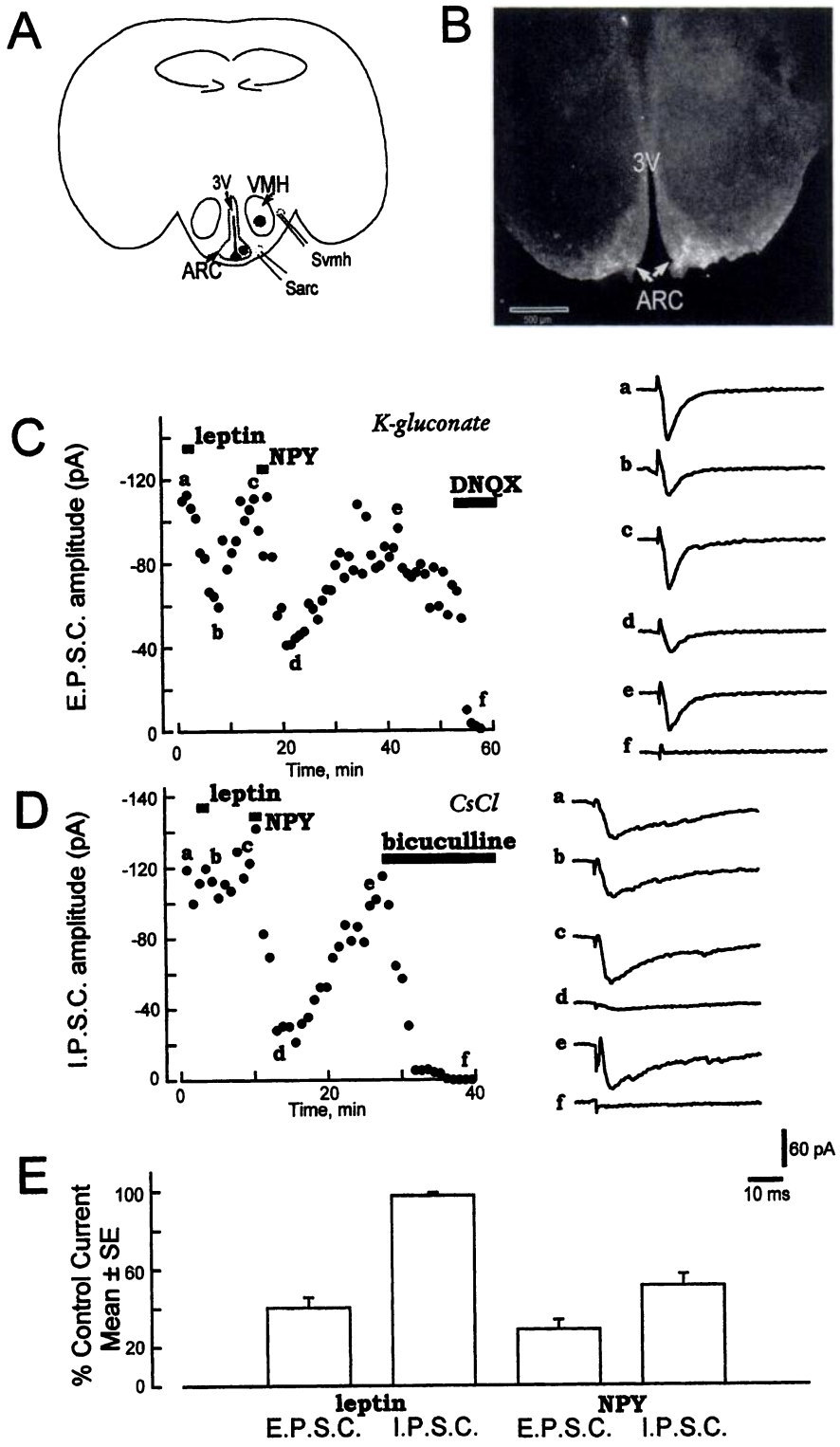

Fig. 2. A, Recording preparation. ARC is indicated surrounding the ventral edge of the third ventricle (*3V*). VMHs and typical sites of neuronal recordings (*shaded regions* within ARC and VMH are illustrated. Stimulation electrodes were placed lateral to the ARC (*Sarc*) and VMH (*Svmh*). *Scale bar*, 500 μm. B, Fluorescence image of immunohistochemical localization of NPY-containing neurons in ARC region from which recordings were made. C, Responses of an ARC neuron to leptin and NPY. At the conclusion of the experiment, the glutamate receptor antagonist DNQX was used to confirm that the EPSC was mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type receptors. *Right*, average synaptic currents at the various time points (*a–f*). D, IPSCs recorded with Cl^--containing electrodes are unaffected by leptin but are potently inhibited by NPY. The addition of bicuculline at the conclusion of the experiment confirms that IPSCs are mediated by γ-aminobutyric $acid_A$ receptors. *Right*, average synaptic currents at the various time points (*a–f*). E, Summary of leptin and NPY effects on evoked EPSCs (15 cells from three rats) and IPSCs (five cells from three rats) recorded in ARC.

ings. In the remaining recordings, incomplete recovery was noted. Repeated application of leptin typically induced a similar degree of inhibition, suggesting a lack of desensitization. In contrast, leptin produced no effect on glutamatergic EPSCs recorded in the VMH (99.3 ± 1.9% control current, three experiments). IPSCs were also unaffected in the VMH (101.5 ± 4.5% control current, three experiments). We studied the effects of NPY on synaptic transmission in the ARC. NPY (250 nM) reduced both the EPSC and IPSC (Fig. 2, C–E).

In addition to its observed effects on synaptic transmission in the ARC, leptin produced a reduction in neuronal input resistance. Leptin produced a ~50% reduction in cell input resistance using K^+-gluconate in current-clamp recordings as measured by brief hyperpolarizing current injections (Fig. 1A). In whole-cell recordings using K^+-gluconate electrodes, leptin increased the whole-cell current, particularly at depolarized potentials (Fig. 1B). The effect of leptin on the whole-cell current was absent in CsCl recordings, which would

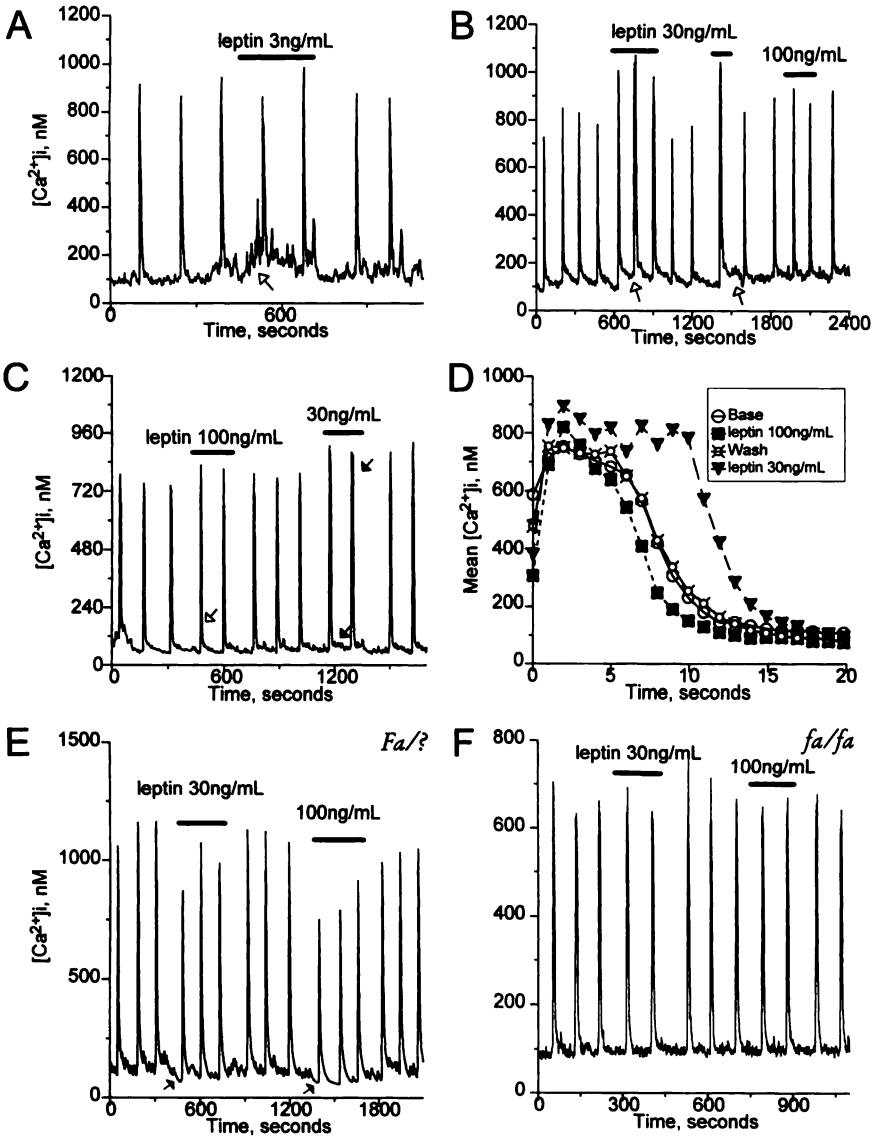

Fig. 3. Leptin affects $[Ca^{2+}]_i$ dynamics in acutely isolated rat ARC neurons. Neurons were isolated from Sprague-Dawley rats (A–D). A, Application of leptin (3 ng/ml with 0.1 mg/ml ovalbumin) produced an increase in basal $[Ca^{2+}]_i$ and spontaneous oscillations (*arrow*). Large spikes in $[Ca^{2+}]_i$ are due to depolarization with 50 mM potassium (50 K^+) every 2 min. B, Application of leptin (30 ng/ml) produced an elevation in basal $[Ca^{2+}]_i$ and an increase in peak width and peak height that was reproducible (*open arrows*). These effects were not produced by 100 ng/ml leptin. C, Application of leptin increased peak height while decreasing peak width (*open arrow*) at 100 ng/ml and increased peak height, basal $[Ca^{2+}]_i$, and peak width (*filled arrows*) at 30 ng/ml. Note that the increase in basal $[Ca^{2+}]_i$ rapidly recovered after leptin washout. D, Higher-resolution plot of average $[Ca^{2+}]_i$ elevations for data shown in C shows a slight decrease in peak width for 100 ng/ml leptin and increase in width for 30 ng/ml leptin. Traces were aligned on the rising phase and numerically averaged. E, Neurons isolated from lean (*Fa/?*) Zucker rat ARC nuclei responded to leptin in a manner similar to that of ARC neurons from Sprague-Dawley rats. Application of 30 ng/ml leptin decreased the peak rise in $[Ca^{2+}]_i$, basal $[Ca^{2+}]_i$, and spontaneous oscillations (*arrow*). Similar effects were seen with 100 ng/ml leptin. Statistical significance was typically obtained for changes in peak area, width, or height (not shown). Overall, responses were observed in each of 13 of 16 cells from five rats at 30 ng/ml and 7 of 10 cells at 100 ng/ml leptin. F, ARC neurons from obese (*fa/fa*) Zucker rats typically failed (9 of 11 cells, six rats) to respond to leptin at 30 or 100 ng/ml. In this example, changes in $[Ca^{2+}]_i$ were not statistically significant ($p > 0.09$) compared with preapplication values.

block K^+ conductances (Fig. 1C). In many cases, the time course of the leptin-evoked current was longer (15–20 min) than its effects on synaptic transmission, indicating that the latter were not mediated by filtering effects of the membrane. We also examined the leptin-evoked current in perforated-patch recordings. ARC neurons demonstrated a slowly developing outward current when stepped from hyperpolarizing to depolarizing potentials (data not shown). After leptin application, the instantaneous current was dramatically increased (264 ± 5.5%, five experiments) and then slowly decreased (Fig. 1D). These data suggest that leptin may affect K^+ conductances.

Zucker fatty (*fa/fa*) rats are believed to be obese because they have a mutation in the leptin receptor (15, 16). Leptin failed to inhibit EPSCs recorded in ARC in slices prepared from obese Zucker (*fa/fa*) rats (Fig. 4). Furthermore, the leptin-induced current was also absent from these cells. However, normal responses were observed in lean (*Fa/?*) littermates (Fig. 4). Interestingly, NPY responses were present in both lean and obese Zucker rats (Fig. 4). Thus, the failure of obese Zucker rats to respond to leptin was a selective phenomenon.

We also searched for the presence of leptin receptors existing postsynaptically on ARC neurons using a nonelectrophysiological paradigm. The basal levels of $[Ca^{2+}]_i$ in many of the dissociated cells oscillated rapidly (e.g., Fig. 3, A and E). When neurons were depolarized with pulses of 50 mM K^+, they exhibited large transient increases in $[Ca^{2+}]_i$. Leptin produced a variety of concentration-dependent effects on both the oscillations of basal $[Ca^{2+}]_i$ and the 50 mM K^+-evoked changes in $[Ca]_i$ in neurons acutely isolated from normal Sprague-Dawley rats. The clearest responses occurred at 30 ng/ml leptin, although responses were sometimes seen at 3 ng/ml, the lowest concentration tested (Fig. 3A). Overall, 35 of 39 neurons showed clear responses to 30 ng/ml leptin within 3 min and typically within 1 min of application. Leptin was frequently observed to increase the time required for $[Ca^{2+}]_i$ to recover after a 2.5-sec K^+ depolarization and also increased the peak attained during the $[Ca^{2+}]_i$ rise (8 of 39 cells) (Fig. 3, B–D) or decreased the peak (6 of 39 cells). An increase in peak width occurred in 15 of 39 cells, whereas a clear decrease in peak width was observed in 7 cells. This decrease in peak width was more consistently seen at 100 ng/ml leptin. The effects of 30 ng/ml leptin were

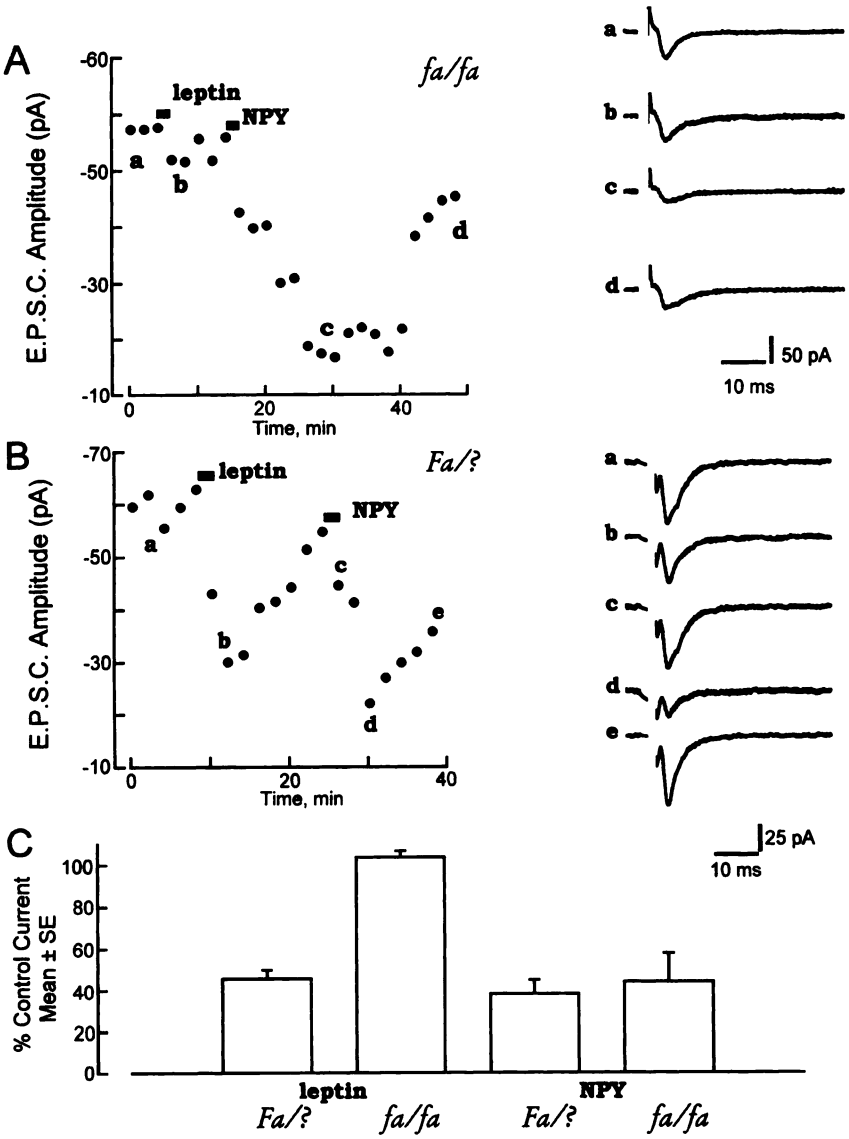

Fig. 4. Leptin (100 ng/ml) failed to significantly reduce evoked EPSCs in ARC in slices prepared from obese Zucker (*fa/fa*) rats (A) but induced normal inhibition in slices prepared from lean (*Fa/?*) controls (B). Responses to NPY (250 nM) are shown for comparison and were not significantly different between obese or lean Zucker and Sprague-Dawley rats. *Right*, time points illustrated (*a–e*). C, Mean inhibition of evoked EPSCs by leptin and NPY in (*fa/fa*) (11 cells/three rats) and (*Fa/?*) (eight cells/three rats) Zucker rats.

repeatable within individual cells, suggesting that there was no rapid rundown or desensitization (Fig. 3B). Recovery was typically obtained within 5 min of leptin removal. Thus, although leptin can produce a variety of effects on $[Ca^{2+}]_i$ (perhaps due to variable membrane potential in dissociated cells), it is clear that leptin affects ARC neurons and may influence more than one ion channel.

A second effect we observed was an increase in oscillation frequency and, perhaps as a consequence, an increase in basal $[Ca^{2+}]_i$ in some cells (11 of 24 cells at 30 ng/ml and 13 of 20 cells at 100 ng/ml). In a few cases, the new plateau level was several hundred nanomoles higher than preapplication and recovery levels (six cells). Typically, no effects of leptin were observed in cells acutely isolated from the VMH (30 ng/ml leptin, six of eight cells).

Interestingly, many dissociated ARC cells (52 of 101 cells) contained NPY, as revealed by immunohistochemical staining and consistent with the abundance of NPY-positive neurons evident within the slice preparation (Fig. 2B).

Cells from lean (*Fa/?*) Zucker rats also displayed responses to 30 ng/ml leptin (13 of 16 cells, five rats). Most often (7 of 16 cells), 30 ng/ml leptin decreased the $[Ca^{2+}]_i$ peak after K^+ depolarization, and the peak was increased in 1 of 16 cases. The peak width decreased in 5 of 16 cases, and oscillations were damped in 2 cases (Fig. 3E). Like the Sprague-Dawley rats, lean Zucker rat ARC neurons showed a decrease in $[Ca^{2+}]_i$ peaks at the 100 ng/ml dose (four of eight cells). In contrast, ARC neurons from obese (*fa/fa*) Zucker rats (six rats) typically failed to respond to 30 (2 of 11 cells), 100 (0 of 6 cells), or 300 (2 of 11 cells) ng/ml. The few "positive" scores involved slight increases in peak width (30 ng/ml leptin; one case at 300 ng/ml) or a slight decrease in peak height (one case at 300 ng/ml). Typically, there were no statistical differences in peak area or width ($p > 0.09$) as a result of leptin treatment (Fig. 3F). In summary, the imaging data suggest that leptin has multiple types of direct effects on ARC neurons in Sprague-Dawley and lean Zucker rats but little or no effect in *fa/fa* Zucker rats.

The results of these experiments show that leptin produces rapid neuromodulatory effects on ARC neurons. The several types of effects we observed may be consistent with the observation of multiple molecular forms of the leptin receptor (11, 12, 16) and perhaps multiple effects produced by activated receptors. These effects would be sufficient to explain

its ability to inhibit the activity of NPY-containing neurons in the ARC, and such an action would contribute to its ability to suppress food intake. The effects of leptin are sufficiently rapid to suggest that changes in gene expression are not necessary to account for its acute actions, although such changes may be linked to those reported here and may be important for its long term effects. It is thought that the NPY-containing neurons originating in the ARC and projecting to the PVN play an extremely important role in the control of feeding behavior (8, 9). It should be noted that in addition to projecting to the PVN, these NPY neurons give rise to terminals that are found within the ARC itself (21). Thus, NPY released within the ARC may influence the activity of NPY-containing ARC neurons in a feedback loop. This would be consistent with the effects of NPY reported here. The results reported suggest that leptin acts by regulating the output of these NPY-containing ARC neurons, a hypothesis that is also consistent with recent neurochemical data (5). Leptin reduces the excitatory input into these cells and produces inhibitory postsynaptic effects. The net result of these actions would be a reduction in the activity of ARC NPY neurons and decreased release of NPY in the PVN and elsewhere. It is therefore possible that the level of food intake is set by the activity of the loop of NPY neurons in the ARC. The level of activity of this loop would be regulated by blood-borne leptin released from fat and sensed by ARC neurons that are at least in part outside of the blood-brain barrier and can bind blood-borne leptin (10, 21). Bypassing the leptin regulatory system by the use of direct injections of NPY into the brain or genetically compromised animals such as obese and diabetic mice and obese Zucker (*fa/fa*) rats would result in excessive feeding behavior. NPY and leptin may also affect neurons and other types of cells in various areas of the brain (10–12, 23), such as those controlling the activity of the sympathetic nervous system; these effects may also have an important impact on feeding behavior.

The results of these experiments also bring into question the mechanism of synaptic modulation produced by leptin. It is known that the receptors for leptin are similar to those of several cytokines including interleukin-6, ciliary neurotrophic factor, and leukemic inhibitory factor as well as the common receptor subunit gp130 (11, 12, 16). These receptors are thought to function through activation of the JAK/STAT pathway, which involves the JAK class of tyrosine kinases. It is not clear how activation of such receptors can produce the rapid effects on synaptic transmission observed in our experiments. Presumably, these are too rapid to require alterations in gene transcription. However, they could involve JAK-mediated phosphorylation of ion channels or similar entities. Indeed, rapid modulation of calcium-activated potassium current in excised membrane patches after activation of the prolactin receptor has been reported, and prolactin receptors are in the same general class of cytokine receptors (24). It will be interesting to see whether the rapid modulation of ion channels in leptin-sensitive neurons occurs by similar mechanisms as observed with other cytokines.

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