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Leptin inhibits swallowing in rats

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Félix, Bernadette, André Jean, and Claude Roman. Leptin inhibits swallowing in rats. *Am J Physiol Regul Integr Comp Physiol* 291: R657–R663, 2006. First published March 30, 2006; doi:10.1152/ajpregu.00560.2005.—Swallowing is under the control of premotoneurons located in the medullary solitary tract nucleus. Although rats with transected midbrain do not seek out food, they are able to ingest food present near the mouth, and acute food deprivation induces an increase in food intake. Leptin is a satiety signal that regulates feeding behavior. Because leptin receptors are found within the caudal brainstem, and because food intake is regulated in midbrain transected rats, this study tested the hypothesis that leptin is able to modify the activity of premotoneurons involved in swallowing. Leptin was microinjected at the subpostremal level of the medullary solitary tract nucleus in anesthetized Wistar rats. Electromyographic electrodes in sublingual muscles allowed recording of swallowing induced by stimulation of sensitive fibers of the superior laryngeal nerve. Repeated stimulation induced rhythmic swallowing. Microinjection of leptin (0.1 pg and 0.1 ng) in the swallowing center induced an inhibition of rhythmic swallowing (latency of <30 s) as shown by the reduced number and strength of electromyographic activities, which could last several minutes. The threshold of the leptin-induced inhibition was close to 0.1 pg. Interestingly, the inhibitory effect of leptin was not observed in leptin receptor-deficient Zucker rats. Here we show that, in Wistar rats, leptin already known to modulate the discharge of medullary solitary tract nucleus-sensitive neurons involved in satiety reflexes can also modify the activity of swallowing premotoneurons, thereby inhibiting an essential motor component of feeding behavior.

leptin; medullary solitary tract nucleus; swallowing; rat

SOLID OR LIQUID EDIBLE food may involve suckling, lapping, mastication, and in all cases swallowing. All of these functions are the motor components of ingestive behavior. The lower brainstem contains all the motoneurons regulating this behavior. During swallowing, the motoneurons are under the control of premotoneurons mainly located within the interstitial and intermediate subnuclei of the solitary tract nucleus (STN) that constitute the so-called “swallowing center” or “central pattern generator” (CPG) of swallowing (SwCPG; see Ref. 23). Although rats with transected midbrain do not seek out food, they will ingest food applied near the mouth, accepting or rejecting food by the taste (16). When they can be kept alive by careful tube feeding, acute food deprivation induces an increase in food intake (17, 18). Ingestive behavior in response to hypoglycemia also occurs in midbrain-transected rats (14).

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The hindbrain contains: 1) several CPGs, the SwCPG but also mastication, suckling or lapping CPGs; 2) the corresponding motoneurons and the preganglionic neurons that control the gut movements, the pancreatic secretion, and the hepatic metabolism; 3) STN neurons that receive, during feeding, positive feedback from the mouth and negative feedback from the stomach, liver, and small intestine. The hindbrain is involved in what may be called the “direct control of eating” (39). In addition, in the intact animal, there are many reciprocal connections between the lower brain and the forebrain, especially the hypothalamus, limbic system, and frontal cortex, i.e., all the structures that are the sources of “indirect control of eating” (39).

Various neurotransmitter systems are involved in swallowing (8, 23). The main results concern induction and facilitation of CPG activity by excitatory amino acids (27), inhibition of the swallowing sequence by GABA (20, 44), and the modulatory influence of monoamines (24). By contrast, little is known on the action of neuropeptides on the SwCPG, in particular on the role of peptides and neurohormones involved in food intake control.

Leptin, the product of the *ob* gene (46), was first identified as a hormone, secreted by adipose tissue, that regulates feeding behavior and energy balance. It was recognized as a satiety signal that acts at the hypothalamic level (11, 21) and reduces body fat by reducing feeding and increasing the catabolic activity of the sympathetic system. It circulates in the blood at levels correlated with body fat mass, which may explain the term of lipostatic signal often employed. Circulating leptin reaches the brain through the choroid plexus by a receptor-mediated transport. Recent data reported that: 1) leptin receptors are found within the caudal brainstem, in the dorsal vagal complex, an area that includes the STN, the area postrema, and the dorsal motor nucleus of the vagus (10, 19); 2) microinjection of leptin (1 μg) in the dorsal vagal complex suppresses food intake (19) measured at 2, 4, and 24 h; 3) leptin is also secreted from gastric mucosa (3), and this gastric leptin is able to increase the activity of STN neurons, the first relay station for vagal afferents (45, 37); and 4) gastric vagal afferent terminals (43) and vagal mechanosensitive fibers from the gastrointestinal tract (15) are responsive to leptin. Therefore, either gastric or adipocyte-derived leptin may act at the level of the STN, directly or via vagal fibers, as a satiety signal reducing food intake (2). Considering the above-mentioned food intake capabilities of mid-brain-transected rats, the question arises as to whether leptin acts uniquely as a signal modulating the discharge of sensitive neurons within the STN

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or if it is, in addition, able to modify directly the activity of STN premotoneurons involved in swallowing. The present work demonstrates that leptin microinjected directly in the interstitial and intermediate STN subnuclei inhibits swallowing, an essential motor component of ingestive behavior, and so participates in the control of food intake and body weight homeostasis.

METHODS

The experimental procedures described here were carried out in accordance with the European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC) and the French law on the Protection of Animals (1987; revised 2001).

Surgical procedures. Experiments were performed on 35 adult Wistar rats weighing from 200 to 350 g, and 12 Zucker rats (6 rats *fal* weighing from 500 to 560 g and 6 rats *fal*+ weighing from 400 to 440 g; Charles River, l'Arbresle, France) anesthetized with a mixture of ketamine (100 mg/ml) and xylazine (15 mg/ml; Centravet, Dinan, France) in a proportion of 90% and 10%, respectively. The anesthesia was then continued by perfusion of the same mixture through a catheter inserted in the peritoneal cavity, at a rate of 0.5–1 ml/h. Each superior laryngeal nerve (SLN) was dissected free from surrounding tissues and placed on miniature bipolar electrodes. Animals were then fixed in a stereotaxic frame (Horsley and Clarke apparatus adapted for rats) in such a position that, after occipitoparietal craniotomy and removal of the posterior part of the cerebellum, the floor of the fourth ventricle appeared to lie in a horizontal plane. Its surface was covered with warm liquid paraffin.

Stimulations and recordings. Swallowing was triggered by stimulation of the sensitive fibers contained in the SLN. Stimulation with a short train of pulses induced a single swallowing, whereas stimulation with a long train of pulses produced several swallows [or rhythmic swallowing recorded by electromyography (EMG)], at a rhythm depending on stimulation frequency (Fig. 1). In the present study, only long trains of pulses (3–5 s duration; 10–30 Hz frequency) were used. The pulse voltage and duration varied according to the animal (1–3 volts; 0.05–0.5 ms). During these stimulations, the respiration was more or less completely blocked (Fig. 1).

To monitor swallowing, the EMG activity of sublingual muscles (mainly the genioglossus) was recorded by means of bipolar copper wire electrodes, insulated except at the tip, and inserted in the muscles (7). In a first series of experiments, the EMG signals, suitably amplified, were displayed on an oscilloscope (Tektronix 5111) and stored on a computer using pCLAMP 6.0.1 software (Axon Instru-

ments, Foster City, CA). In a second series, we had at our disposal a PowerLab data acquisition system with eight input channels connected to a personal computer, with chart software for Windows (record, display, store, analyze). Body temperature was recorded continuously by a rectal thermal probe and was maintained at 36–37°C by means of a warming device. Electrocardiogram (ECG) picked up by subcutaneous electrodes on each side of the thorax, and respiratory activity recorded by a mechanotransducer (or a thermistance in front of the nose) allowed heart rate (ECG) and respiration monitoring, respectively (see Fig. 1). Electrocardiogram and swallowing EMG fed loud speakers for auditory control.

Microinjections. Pressure ejections of drug solutions were performed, either directly through a Hamilton syringe allowing the injection of 50–100 nl or via glass pipettes (70–100 μ m OD at the tip) using a pneumatic pressure system (Neurophore BH2; Medical System, Great Neck, NY). The following agents, obtained from Sigma, were used: 6×10^{-9} to 6×10^{-12} M leptin, 10^{-3} to 10^{-9} M L-glutamic acid, and 10^{-3} M γ -aminobutyric acid (GABA). All of these drugs were dissolved in a 0.9% NaCl solution. In a first series of experiments, high doses of leptin were used to search for a possible effect of the drug. Next, lower doses were used to determine the threshold of leptin-induced action.

Recombinant rat leptin was first dissolved in 1 ml distilled water. The solution was diluted in PBS, separated into aliquots, and stored at -70°C . One aliquot (1 μ l) was used for each experiment.

The maximum volume ejected through the micropipette was 50 or more frequently 100 nl (amount measured with the Hamilton syringe used to inject the drug in the glass pipette). For pressure ejection, the volume was checked under a microscope, and the parameters (between 80 and 150 kPa for pulses of 1–5 s duration) were adjusted to obtain 100 nl of leptin colored by addition of pontamine blue in a droplet of paraffin oil. Therefore, the maximum amount injected in the structure was 0.1 pg or 0.1 ng.

Histological controls. In 20 experiments, pontamine blue (4%) was added to the drug solution injected. It had previously been checked that this dye alone produced no effect on swallowing. At the end of these experiments, the brainstem was removed and placed in a solution of 4% formaldehyde. Frontal frozen sections (30–50 μ m thick) were cut and examined for histological localization of the injection site.

Experimental procedures. We studied how various agents [leptin, glutamate (swallowing agonist), GABA (swallowing antagonist), and vehicle of drugs alone (0.9% NaCl)] affect the rhythmic swallowing elicited by long repetitive stimulations of the SLN. The drugs were microinjected stereotaxically in the SwCPG, located in the region of STN extending between 0.5 and 0.7 mm rostral to the caudal edge of the area postrema (taken as the 0), 0.6–0.8 mm laterally, and 0.6–0.8 mm in depth. These coordinates correspond to those given by Kessler et al. (25–27) for rats of similar weight. In addition, glutamate was also tested in the absence of SLN stimulation to check whether the drug triggered swallowing when the micropipette was positioned according to the above-mentioned coordinates.

After the positioning of the micropipette in the injection site, stimulation trains of pulses were delivered to the ipsilateral SLN every 30 s. A control episode including at least five trains of stimulations was performed before drug injection. Next, stimulation and recording were maintained until recovery.

Statistical analysis. Cardiac and respiration frequencies were expressed as means \pm SE. Data were analyzed using ANOVA, followed by Fischer's protected least-significant difference test. Statistical analysis software (StatView for windows 5.0.1; SAS Institute) was used. The criterion for significance was $P < 0.05$.

RESULTS

Checking the localization of the SwCPG. To check that the stereotaxic coordinates used in these experiments corresponded to those of the SwCPG, we have shown that, in accordance

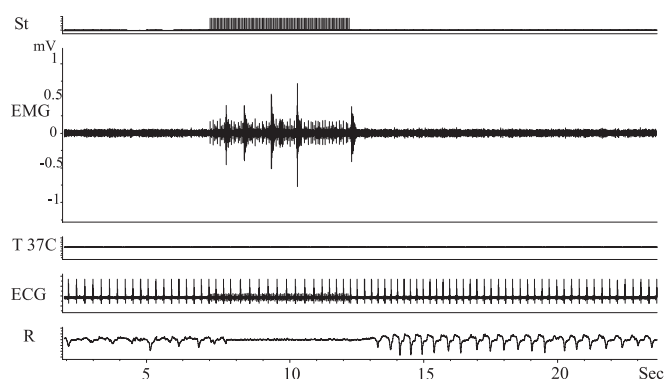


Fig. 1. Example of polygraphic recording. St, stimulation of SLN at 20 pulses/s during 5 s (pulse parameters, 1.5 volts and 0.2 ms); EMG, electromyogram from genioglossus muscle. Note the rhythmic swallowing triggered by superior laryngeal nerve (SLN) stimulation; T, rectal temperature in $^{\circ}\text{C}$; ECG, electrocardiogram; R, respiration. Note the respiration blockade during the stimulation inducing rhythmic swallowing.

with previous demonstration (26), microinjection of glutamate within this site can initiate swallowing (Fig. 2A), as does SLN stimulation. The effects of microinjections of leptin were tested at the same site. In addition, we controlled that GABA injected at this site (Fig. 2B) induced an inhibition of swallowing (8, 44). In some experiments ($n = 12$), we injected, at the same site, glutamate and then leptin. The coronal section of Fig. 2C indicates the area within which all injections of drugs were made.

Effects of leptin microinjections in the SwCPG. In 51 trials performed on 31 Wistar rats, microinjections of leptin, either at low (13 rats) or high doses (18 rats), always resulted in an inhibition of rhythmic swallowing (Fig. 3). The effect occurred with a very brief latency (Fig. 3A). Indeed, the number of swallows usually decreased as soon as the first train of stimulating pulses after microinjection. Considering that stimulations were repeated every 30 s, and that injection was made between two of them, it can be deduced that the latency was <30 s. The duration and intensity of this inhibition varied according to the doses of leptin injected. At the lowest doses (0.1 pg or 6×10^{-12} M), inhibition lasted 5–7 min at the most and only occasionally resulted in a total suppression of rhythmic swallowing (Fig. 3A1). At higher doses (0.1 ng or 6×10^{-9} M), the effects were more pronounced; not only did rhythmic swallowing usually disappear, but also, the duration of the inhibition ranged from <15 min up to 1 h and more. Partial recovery was only obtained in a few experiments when higher doses were used. One example is shown in Fig. 3B. The intensity of inhibition could be evaluated according to the following two criteria: number of swallows per pulse train and strength of individual swallowing contractions before the total disappearance (when occurring). As indicated above, we always observed a decrease in the number of swallows (Fig. 4), and very frequently their disappearance. The decrease in the number of swallows was accompanied by a reduction in the intensity of the global swallowing EMG, more especially

visible when the inhibition was powerful (Fig. 3B). The inhibitory effect of leptin was similar to that obtained with GABA microinjection. It is worth noting, however, that recovery was more rapid after GABA injection than after leptin application (Fig. 4).

During the inhibitory action of leptin, and particularly when rhythmic swallowing disappeared, a further increase in stimulation parameters allowed the swallows to reappear or to increase in number (Fig. 3B), indicating that the SwCPG excitability had been reduced, with an increase of its triggering threshold. This result was not because of lesions of afferent fibers or changes in electrode impedance, since, after recovery, the stimulation parameters for obtaining control rhythmic swallowing returned to initial values.

As for cardiac and respiratory rhythms, no changes were observed under the action of leptin (Fig. 4). The well-known inhibition of respiration (9) during swallowing afferent stimulation also persisted (Fig. 1).

Control experiments. Intramedullary microinjections of 0.9% NaCl or pontamine blue solutions (trials on 3 different rats) caused no effect on rhythmic swallowing, even after several applications. It was also worth noting that the inhibitory effect of leptin was observed only when the ipsilateral SLN was stimulated. Stimulation of the contralateral SLN still induced swallowing (data not shown).

To evaluate the specificity of the effect of leptin, a series of microinjections was performed on genetically obese Zucker rats, with the lowest doses of leptin (0.1 pg) that range within the physiological concentrations (see DISCUSSION). No modification of swallowing activity was observed when leptin was microinjected in STN of homozygous rats (*fa/fa*; Fig. 5, A and C). Interestingly, within the same sites, GABA microinjection (10^{-3} M) still induced clear-cut inhibition of swallowing (Fig. 5B). A slight but not significant decrease of swallowing number was obtained when leptin microinjections were performed in heterozygous (*fa/+*) Zucker rats (Fig. 5C).

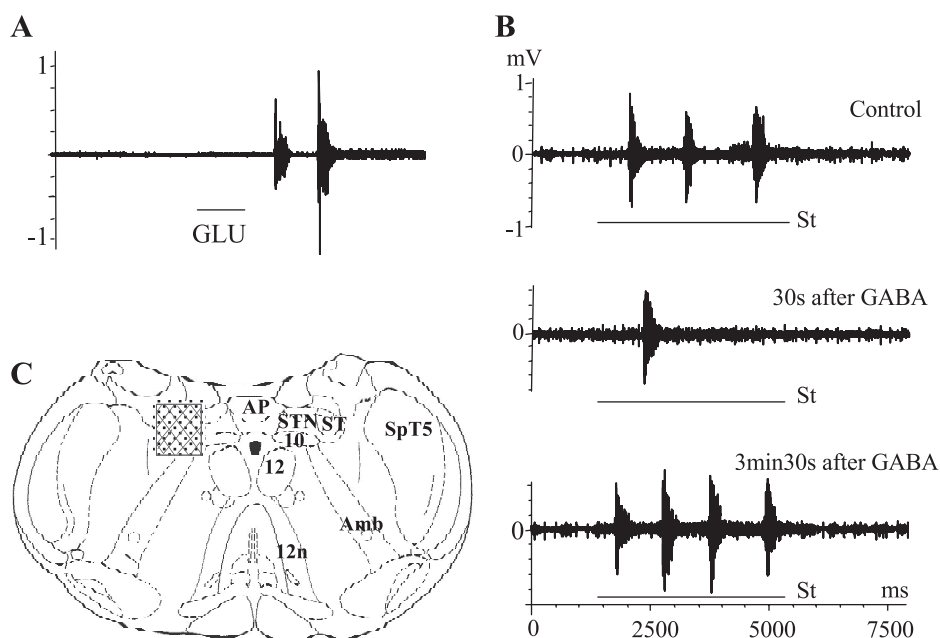


Fig. 2. Localization of the central pattern generator of swallowing (SwCPG). The 2 experiments in A and B were carried out on 2 different rats. The coronal diagram of the medulla in C, drawn from the atlas of Paxinos and Watson (35a), corresponds to the level of the injection sites of glutamate, γ -aminobutyric acid (GABA), and leptin (between 0.5 and 0.7 mm in front of the caudal edge of the area postrema). Although microinjections were made on both sides of the medulla, all sites of injections have been reported to the left side and are gathered in the hatched area corresponding to the region of the interstitial and intermediate solitary tract nucleus (STN) subnuclei. Amb, ambiguous nucleus; AP, area postrema; SpT5, spinal trigeminal tract; ST, solitary tract; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus; 12n, roots of hypoglossal nerve. A: microinjection of glutamate (GLU, 10^{-6} M; 1 pressure pulse); 2 swallows were triggered. B: effect of microinjection of GABA (10^{-3} M; 1 pressure pulse) on rhythmic swallowing. SLN repetitive stimulation (St, 5 volts, 0.06 ms, 15 Hz during 4 s). Note the inhibition of rhythmic swallowing after GABA injection and the recovery with a rebound effect.

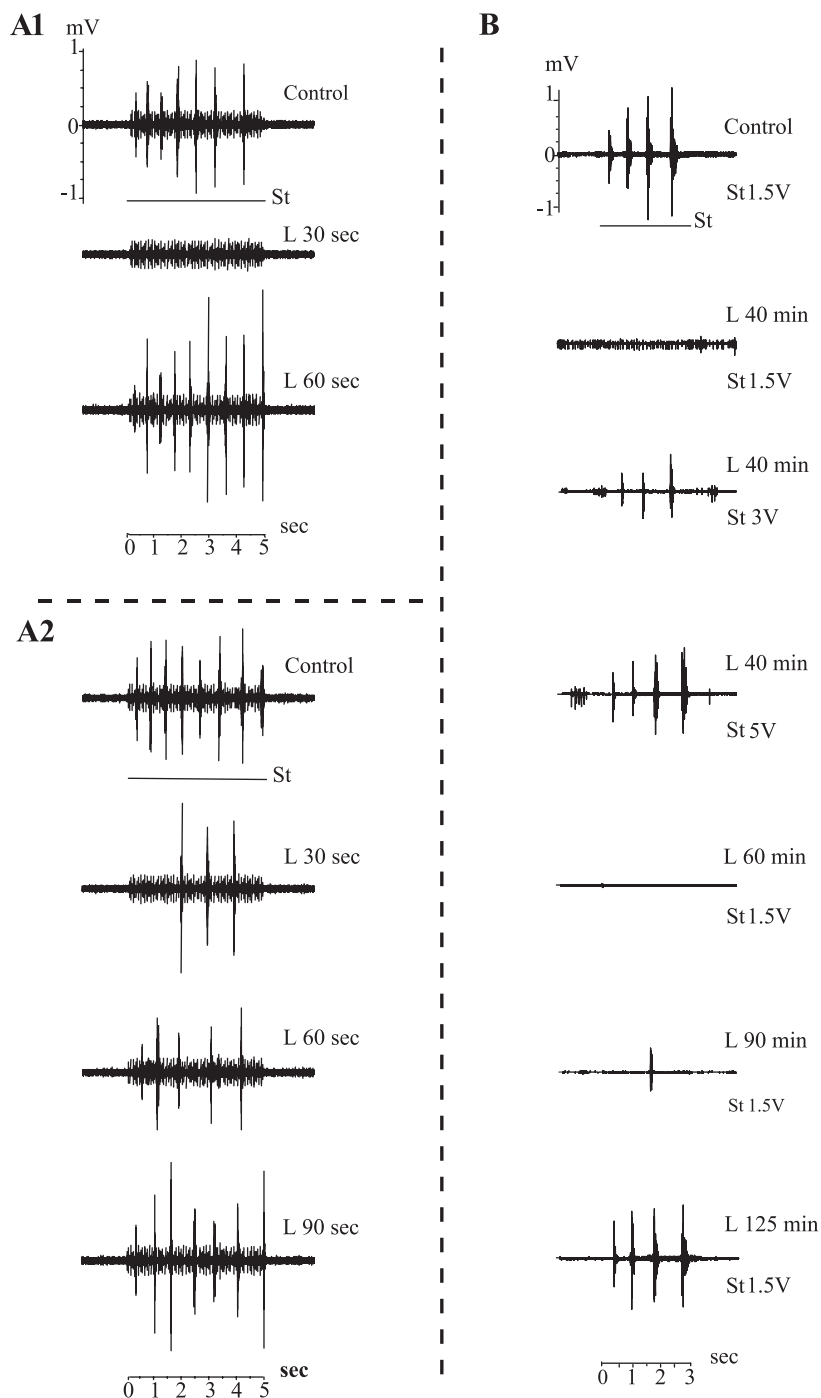


Fig. 3. Leptin microinjections in the medullary STN inhibit rhythmic swallowing triggered by SLN stimulation; effects of the doses. Tracings in *A* and *B*, obtained from 2 different animals, should be read from *top* to *bottom*. The leptin concentration was 0.1 μ g for *A1* and *A2* and 0.1 ng for *B*. Stimulation parameters in *A1* and *A2* were 5 volts, 0.2 ms, 20 Hz during 5 s. Note in *A1* the complete and very short inhibition of rhythmic swallowing and, in *A2*, the less important inhibition and the slightly longer recovery. In *B*, the stimulation voltage was different, as shown on the tracings. For the first (control) and the last (recovery) tracings, the parameters were as follows: 1.5 volts, 0.08 ms, 30 Hz during 3 s. For all tracings, the voltage is indicated under the trace, with the other parameters remaining identical. Note 1) that the inhibition of rhythmic swallowing was powerful (disappearance of the response) and long-lasting (recovery after 125 min) and 2) that, during maximum inhibition, between 40 and 60 min, the increase in voltage stimulation was allowed to obtain a rhythmic swallowing very similar to that of control, indicating that during this period the swallowing CPG was less excitable but nevertheless able to function. L 30 sec to L 125 min indicates the time after leptin injection.

DISCUSSION

This study showed that leptin inhibits swallowing, a fundamental component of food intake. This result is congruent with the well-documented action of leptin on ingestion (2, 5, 6, 12, 13). What was new, however, is the fact that this adiposity signal, strongly involved in satiety, was able to act not only on the afferent and integrative system of feeding but also on the motor component of this system. This is probably one explanation for the capacity for food intake regulation in the mid-brain-transected animals (18).

Within the brainstem site studies, the leptin-induced effect on swallowing appears to be specific, since inhibition was

never observed with 0.9% NaCl or pontamine blue solutions, and leptin had no effect on heart rate and respiratory rhythm. In addition, in the leptin receptor-deficient rat Zucker, leptin did not modify swallowing activity, whereas GABA still induced an inhibitory effect.

The fact that leptin was injected within the SwCPG area could be attested by the following evidence: 1) microinjections of transmitters (glutamate and GABA) known to be involved in swallowing activation or inhibition, respectively (23, 25, 20), gave the expected responses; 2) in the experiments where injections of glutamate and leptin were made successively at the same point, the results were those ex-

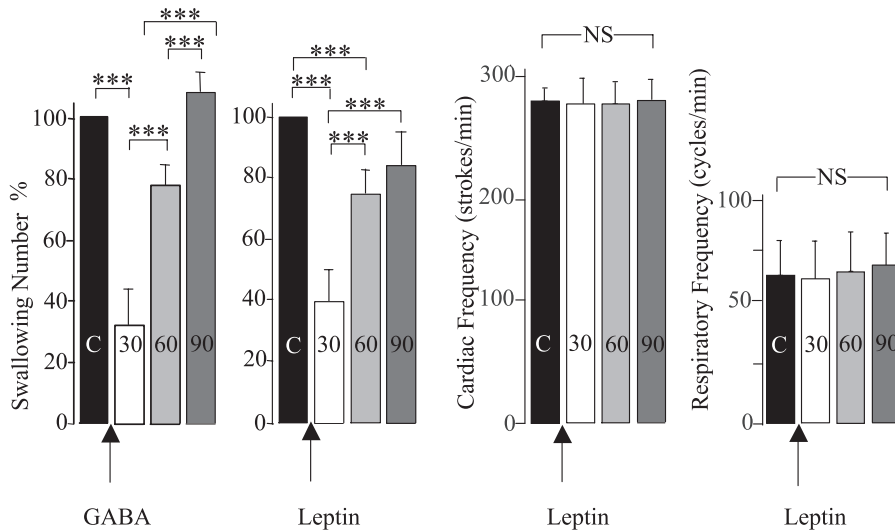


Fig. 4. Effects on swallowing of leptin and GABA microinjections in the medullary STN. Each bar represents the percentage of the nos. of rhythmic swallowing or mean frequencies of cardiac or respiratory rhythms during a period of time of 30 s before [control (C)] and after (30, 60, 90 s) injections of 0.1 pg of leptin (L) or GABA (10^{-3} M), indicated by arrows. Leptin and GABA significantly inhibit rhythmic swallowing triggered by SLN stimulation ($***P < 0.001$). Note a more rapid recovery after GABA than after leptin injection. In addition, it could be noted that no significant difference (NS) in the frequencies of cardiac and respiratory rhythms were observed.

pected (excitation and inhibition, respectively); and 3) the latency of leptin effect was very short (< 30 s), indicating that the drug must have been injected very close to the neurons of the SwCPG.

Variations in leptin effects could be explained by the quantity of drug injected (0.1 pg or 0.1 ng). The major difference between the two doses concerned the duration of the effects obtained, which is easy to understand since it was with the

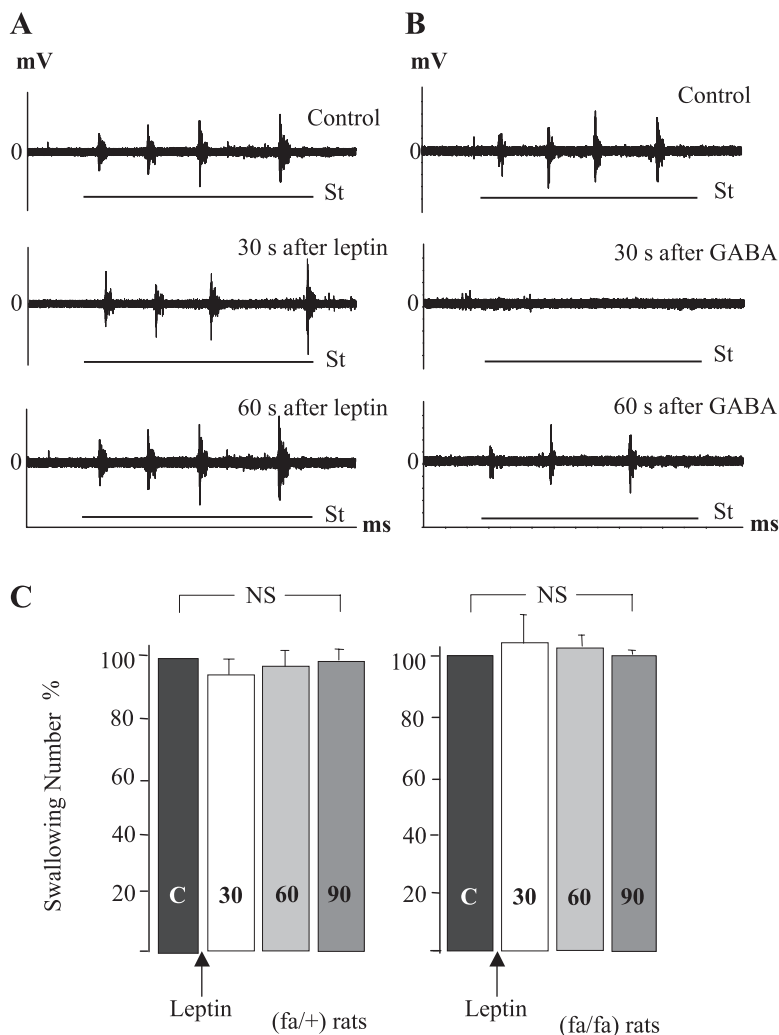


Fig. 5. Effect of leptin and GABA microinjections within the medullary STN of genetically obese Zucker rats. Effect of leptin (A) and GABA (B) microinjections performed within the same site of homozygous (*fa/fa*) Zucker rats. Note the typical inhibitory effect of GABA (10^{-3} M), whereas leptin administration induced no modification in rhythmic swallowing. C: effect of leptin microinjections in heterozygous (*fa/+*; $n = 19$) and homozygous (*fa/fa*; $n = 17$) Zucker rats. Each bar represents the percentage of the nos. of rhythmic swallowing during a period of time of 30 s before (C) and after injection (30, 60, 90 s) of 0.1 pg of leptin, indicated by arrows.

higher dosage that the duration of the inhibition showed greater variability. Blood leptin concentrations in normal rats are in the range of 2–10 ng/ml (0.1–0.6 nM; see Refs. 1 and 41), with an increase of ~2 ng/ml (0.1 nM) after feeding. Leptin can readily cross the blood-brain and blood-cerebrospinal fluid (CSF) barriers to reach the central nervous system (4, 29), but the mechanisms of this transfer are still a matter of debate, although it seems to be acknowledged that high-affinity transport systems, some being saturable, are involved (4, 29). Some reports in humans (28, 31) and sheep (42) mention that the amount of leptin in the CSF is lower than that in the blood, with the CSF-to-plasma leptin ratio being near 0.025. If this ratio is of same magnitude in the rat, the maximum CSF leptin concentration should be in the range of 0.05–0.25 ng/ml (2.5×10^{-11} to 1.5×10^{-12} M). The lowest doses employed in our experiments (6×10^{-12} M) are very close to these values and so fall within the physiological range. On the contrary, the highest doses (6×10^{-9} M) used in the initial experiments for detecting a possible action of leptin belong to the pharmacological domain. It must be emphasized that, in works using the patch-clamp technique on hypothalamic slices, leptin is currently perfused at 10^{-6} to 10^{-11} M (30, 36, 38, 40).

Leptin receptors are abundant in the lower brain, and particularly in the dorsal vagal complex, which includes the STN (18, 22). Numerous data gathered over 30 years in the laboratory have demonstrated that the interstitial and intermediate STN subnuclei are an essential part of the SwCPG, i.e., the one that programs the entire motor sequence of swallowing (23, 27). It is thus tempting to assume that leptin decreases the excitability of this programming system, which is able to function rhythmically when solicited by repetitive afferent stimulations (rhythmic swallowing). Our study does not allow us to demonstrate the mechanism of leptin action that might consist in postsynaptic inhibition of the neurons themselves or presynaptic inhibition of excitatory afferent fibers. However, in the medial part of the arcuate nucleus of the hypothalamus slices from Wistar rats, it was shown that leptin inhibited ~60% of neurons through a direct postsynaptic action (32). In addition, some works indicate that the inhibitory action of leptin may involve GABA (35).

The inhibitory action of leptin on the STN premotoneurons reported in this study is not in accordance with that described by other authors who worked on the sensitive neurons of the STN, relaying the vagal fibers issued from the stomach (37). Those authors observed a facilitation of neuronal discharge evoked by stomach loading, which is in keeping with the satiating role of stomach distension and of leptin injection. Yet, it cannot be taken for granted that all of the STN neurons behave in the same way. Some visceral afferent fibers of the vagus nerve, having a role in satiation, decrease firing when satiety occurs. It is the case, for example, of the glucosensitive fibers coming from the liver, which stop firing when glucose is infused in the portal vein (33, 34). It has been shown that the resting discharge of such glucose-sensitive fibers was inhibited by peripherally acting leptin (38). It is reasonable to envisage that the STN neurons connected to these fibers should also be inhibited by circulating leptin. In fact, the mode of action of leptin, like that of many other transmitters, probably varies according to the functional context or purpose.

In conclusion, our results show that leptin at physiological doses inhibits the SwCPG in anesthetized rats and conse-

quently the activity of the motoneurons involved in swallowing. Because this motor activity has a major role in ingestion, it can therefore be inferred that leptin inhibits ingestion by acting not only on the afferent and integrative components of the feeding nervous system but also on its motor outputs.

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