BDNF/TrkB signalling interacts with GABAergic system to inhibit rhythmic swallowing in the rat.

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ABBREVIATIONS

ANOVA: analysis of variance
AP: area postrema
BDNF: Brain-derived neurotrophic factor
CCK: cholecystokinin
CPG: central pattern generator
Ctrl: control
DMNX: dorsal motor nucleus of the vagus
DMSO: dimethylsulfoxide
DVC: dorsal vagal complex
ECG: electrocardiogram
ELISA: Enzyme-Like ImmunoSorbent Assay
EMG: electromyogram
GABA: γ-aminobutyric acid
ns: non significant
NTS: nucleus tractus solitarii
PVN: paraventricular nucleus
Resp: respiration
SLN: superior laryngeal nerve
Stim: stimulation
SwCPG: swallowing central pattern generator
TrkB: Tropomyosin-related kinase receptor type B
VMH: ventromedial hypothalamic nucleus
ABSTRACT

Brain-derived neurotrophic factor (BDNF) acts as an anorexigenic factor in the dorsal vagal complex (DVC) of the adult rat brainstem. The DVC contains the premotoneurons controlling swallowing, a motor component of feeding behavior. Although rats with transected midbrain do not seek out food, they are able to swallow and to ingest food. Because BDNF and Tropomyosin-related kinase B receptors (TrkB) are expressed in the DVC, this study hypothesized that BDNF could modify the activity of premotoneurons involved in swallowing. Repetitive electrical stimulation of the superior laryngeal nerve (SLN) induces rhythmic swallowing that can be recorded using electromyographic electrodes inserted in sublingual muscles. We show that a microinjection of BDNF in the swallowing network induced a rapid, transient and dose-dependant inhibition of rhythmic swallowing. This BDNF effect appeared to be mediated via TrkB activation, since it no longer occurred when TrkB were antagonized by K-252a. Interestingly, swallowing was inhibited when subthreshold doses of BDNF and GABA were coinjected, suggesting a synergistic interaction between these two signalling substances. Moreover, BDNF no longer had an inhibitory effect on swallowing when coinjected with bicuculline, a GABA<sub>A</sub> receptor antagonist. This blockade of BDNF inhibitory effect on swallowing was reversible, since it reappeared when BDNF was injected 15 minutes after bicuculline. Finally, we show that stimulation of SLN induced a decrease in BDNF protein within the DVC. Taken together, our results strongly suggest that BDNF inhibits swallowing via modulation of the GABAergic signalling within the central pattern generator of swallowing.
INTRODUCTION

Increasing attention is being focused on various neuropeptides for their ability to regulate functions related to food intake. Among them, the brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has recently attracted attention because it is involved in the central regulation of food intake, acting as an anorexigenic factor in the adult rodent. Indeed, infusion of BDNF in the lateral ventricles induces a decrease in food intake and weight loss in rats (32). Moreover, mice heterozygous for targeted disruption of BDNF, as well as conditional BDNF mutants, show hyperphagia and obesity (21, 36). BDNF is abundantly expressed in the adult brain, and more especially in the hypothalamus and caudal brainstem (4), which are both important structures to maintain normal weight and play major roles in the regulation of energy homeostasis. However, most studies so far have mainly focused on the hypothalamus. Xu et al. (44) demonstrated that BDNF expression in the ventromedial hypothalamic nucleus (VMH) is regulated by nutritional state. In addition, Wang et al. showed that BDNF injections within the VMH or the paraventricular nucleus of the hypothalamus (PVN) induce a decrease in food intake and body weight (40-42). Focusing on the brainstem, we recently showed that BDNF also exerts its anorexigenic effects within the dorsal vagal complex (DVC). Indeed, infusion of exogenous BDNF within the DVC induces anorexia and weight loss. Moreover, in the DVC, the BDNF protein content is modulated by nutritional state and increased by anorexigenic hormones cholecystokinin (CCK) and leptin (2). Although BDNF protein can bind to two structurally unrelated plasma membrane receptors (31), the low-affinity p75 receptor and the high-affinity Tropomyosin-related kinase receptor type B (TrkB), only the latter has been shown to be involved in BDNF anorexigenic effects at the central level. Mice with overall reduced expression of TrkB show hyperphagia and excessive body weight (44). In the hypothalamus, the feeding inhibition induced by BDNF injection within the VMH was attenuated by pretreatment with a TrkB-Fc fusion protein that blocks binding of BDNF with TrkB (42). In the DVC, TrkB receptors are highly expressed (45), however their involvement in anorexigenic BDNF effects remains to be determined.

The DVC, located dorsally in the caudal brainstem, comprises the area postrema (AP), the nucleus tractus solitarii (NTS) and the dorsal motor nucleus of the vagus nerve (DMNX). The DVC is an integrative brainstem center for autonomic functions, for example integrating satiety signals so as to adjust meal size as a function of fat stores (25). However, the role of the DVC in food intake control is not limited to the integration of peripheral signals; it is also involved in the programming of the motor component of feeding behavior. Thus, rats with fully
transected midbrain do not seek out food but are able to ingest food applied in the mouth and to terminate a meal by ceasing to ingest as shown by food dribbling from the mouth. Moreover, these rats are able to adapt their meal size in function of the energy content of food (12-14). In addition, it has been clearly established that the DVC contains the neural network that forms the central pattern generator of swallowing (SwCPG). It has been shown that sensory afferent fibers travelling through the superior laryngeal nerve (SLN), a branch of the vagus nerve that plays an important role in triggering swallowing, contact premotoneurons. These premotoneurons are located within the interstitial and intermediate subnuclei of the NTS that constitute the so-called “swallowing center” or “SwCPG” (19). Interestingly, it has recently been shown that local application of the anorexigenic hormone leptin within the NTS can modulate the act of swallowing (9), which is a fundamental motor activity that constitutes the last step of ingestive behavior (19).

Since i) BDNF is highly expressed in the DVC where it plays a role as an anorexigenic factor, ii) the SwCPG is mainly located in the DVC, iii) leptin microinjection in the DVC inhibits swallowing, and iv) leptin modulates BDNF protein content within the DVC, we hypothetized that BDNF may also modulate the swallowing reflex in the rat.

γ-aminobutyric acid (GABA), a well-known neurotransmitter in the brain, is also known to induce a rapid, transient and dose-dependant inhibition of rhythmic swallowing when injected within the NTS (9, 43). Actually, in physiological conditions, swallowing appears to be subject to a tonic GABAergic inhibition (43). Interestingly, various data have shown that BDNF affects GABAergic signalling within the brain (20, 28, 29, 33).

In consequence, the present study was performed to i) evaluate the actions of BDNF and TrkB receptors on swallowing, ii) determine whether GABA can mediate putative BDNF effects on swallowing, iii) determine whether the endogenous BDNF protein content in the DVC is sensitive to the SLN stimulation paradigm used to induce swallowing.
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).

1) Surgical procedures
Experiments were performed on 44 adult male Wistar rats weighing from 250 to 350 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. The superior laryngeal nerve (SLN) was dissected free from surrounding tissues and placed on miniature bipolar electrodes. The head of the animals was 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. The surface of the medulla was exposed in order to allow the stereotaxic introduction of the microelectrode in the vicinity of the NTS and was covered with warm liquid paraffin.

2) Stimulations, recordings and signal analysis
Swallowing was triggered by peripheral stimulation of the sensitive fibres contained in the SLN. Stimulation with a long train of pulses produced several swallows [or rhythmic swallowing recorded by electromyography (EMG)], at a rhythm depending on stimulation frequency. In the present study, repetitive long trains of pulses (5 s duration at 5-30 Hz frequency every 30 s) were used. The pulse voltage, duration and frequency varied according to the animal (1.5-5 V; 0.02-0.5 ms) in such a way as to trigger 4-6 rhythmic swallows. To monitor swallowing, the EMG activity of sublingual muscles (mainly the geniohyoïd) was recorded by means of bipolar copper wire electrodes inserted into the muscles, using a hypodermic needle. An electrocardiogram (ECG) picked up by subcutaneous electrodes on each side of the thorax, and respiratory activity recorded by a mechanotransducer placed around the thorax, were monitored. Moreover, the electrocardiogram and swallowing EMG signals were fed to loud speakers for auditory monitoring. Rectal temperature was monitored and maintained around 37°C with a heating pad. The EMG, ECG and respiration signals were recorded on a computer using an analog-to-digital interface (PowerLab 8SP data acquisition with Chart5.5 software for Windows,
ADInstruments, USA). EMG activity triggered by the five-second SLN stimulation was analyzed using a specially-designed computer program. This analysis allowed us to determine 1) the number of swallows triggered by SLN stimulation, and 2) the mean area under the envelope of EMG signals (obtained by an average of 8 ms buffers smoothed by using a low-pass filter; time constant = 24 ms) recorded during SLN stimulation. This last value expressed as arbitrary units gives a good representation of swallowing intensity. Cardiac and respiratory frequencies were analyzed continuously during the experiment. All calculated values were normalized as percents of those generated from control values.

3) **Microinjections**
Pressure ejections of drug solutions were performed through glass pipettes (70 μm O.D. at the tip) using an injection device (PMI-200, Dagan Corp., Minneapolis, MN USA). The pressure ejection was adjusted between 150 and 200 kPa for pulses of 3-5 seconds in duration, and the injected volume was 50-100 nl. The following substances, obtained from Sigma (Saint Quentin Fallavier, France), were used: BDNF (7.2, 36 or 72 fmol), bicuculline (250 or 500 pmol), γ-aminobutyric acid (GABA 0.1 fmol, 0.1 pmol or 0.1 nmol). All these drugs were dissolved in NaCl 0.9% solution. K-252a (25 fmol) obtained from Calbiochem (VWR International, SAS, France) was first dissolved in 100% dimethylsulphoxide (DMSO), then in NaCl 0.9%.

4) **Histological controls.**
In 15 experiments, pontamine blue (4%) was added to the drug solution injected. It had previously been ascertained that this dye alone produces 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 (50 µm thick) were cut and examined for histological localization of the injection site.

5) **Experimental procedures**
We studied how various drugs [BDNF, GABA, bicuculline (GABA<sub>A</sub> antagonist), K-252a] affect the rhythmic swallowing elicited by repetitive stimulations of the SLN. Previous studies have shown that a microinjection of glutamate within the intermediate NTS, containing the SwCPG, can initiate swallowing (9, 22, 23). Thus, glutamate microinjections (1 fmol) were used as a control to check that microelectrodes were positioned within the SwCPG. When such a glutamate microinjection elicited swallowing, the stereotaxic coordinates were conserved for BDNF, GABA, bicuculline and/or K-252a injections. The precise coordinates extended between
0.5-0.7 mm rostral to the caudal edge of the AP (taken as the 0) 0.6-0.8 mm laterally and 0.4-0.8 mm in depth, roughly corresponding to those previously used (9). The stimulation was applied to the ipsilateral SLN to test the effects of various drugs on swallowing, and was applied to both SLNs to test the effect of SLN stimulation on endogenous BDNF protein content at the DVC and hypothalamus levels. A control sequence involving three trains of stimulations of 90 s was performed before drug injection. The mean values obtained during this sequence were used as control values. Afterwards, stimulations and recordings were maintained until recovery.

6) DVC and hypothalamus dissection
Endogenous BDNF protein content was measured in the DVC and for comparison, in the hypothalamus of the same rats after SLN bilateral stimulation (performed from 02:00 PM to 4:00 for each rat, n = 5). Control animals (n = 5) were operated at the same time but no stimulation was applied. After two hours the rats were decapitated. Each brain was removed rapidly from the skull and split by a transverse cut between cerebral hemispheres and cerebellum. The hypothalamus was dissected from the forebrain under binocular control (Niko SMZ-2B; Fabre mesurelec s.a.). The brainstem-upper cervical spinal cord block was placed on a tissue chopper (McIlwain Tissue chopper; The Mickle laboratory engineering Co. Ltd) and serially sectioned into 500 µm-thick slices. DVC was microdissected from three relevant slices under binocular control in ice-cold saline. Tissue samples (DVC and hypothalamus) were immediately placed in microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C until use. For each rat, the total amount of time from sacrifice to sample freezing was under 10 minutes. In order to rule out interference from the known circadian variation in BDNF expression (34), two rats (one stimulated vs one non-stimulated) were processed simultaneously every day for five days.

7) Protein extraction and dosage
Tissue samples were homogenized in lysis buffer (100 µl for DVC, 500 µl for hypothalamus) [NaCl 137 mM; Tris-HCl 20 mM; Triton X-100 1%, glycerol 10%, sodium orthovanadate 1 mM, phenylmethylsulfonyl fluoride 1 mM, protease inhibitors cocktail (Sigma)], incubated 30 min at 4°C and centrifuged at 10 000 x g (30 min at 4°C). The supernatants were collected and protein concentration determined using the BCA Protein assay Kit (Novagen, Darmstadt, Germany), according to the manufacturer’s instructions.
8) BDNF immunoassay

BDNF protein content was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Promega, Charbonnière, France) according to the manufacturer’s instructions. The detection limit of the assay is 15 pg/ml and the cross-reactivity with other related neurotrophic factors is less than 3%. For each sample, 30 µg of total proteins were used to determine BDNF content. The within-assay variability was less than 3% and all relevant comparisons were made within the same assay. BDNF concentration was determined as picograms per milligram of total protein, and relative changes in BDNF content between nervous structures of stimulated rats expressed as percentage of sham values.

9) Statistical analyses

Statistical analyses were performed using analysis of variance (two-way ANOVA) followed by Fisher’s protected least-significant difference test (StatView for Windows 5.0.1; SAS Institute). Data were expressed as mean ± SEM. Differences were considered significant when $P < 0.05$. 

RESULTS

Peripheral afferent stimulation triggered reflex swallowing

Repetitive electrical stimulation of the SLN branch of the vagus (2.5 V; 0.6 ms; 20 Hz, 5 s every 30 s) elicited rhythmic swallowing (Fig. 1A). The number (4.87 ± 0.08; n = 81) and the mean intensity of swallows (102.44 ± 9.41 arbitrary unit) triggered by SLN stimulation were stable throughout the experiment. At the same time as this rhythmic swallowing, there was a total cessation of respiration.

BDNF microinjections within the SwCPG inhibited swallowing

The present results, obtained from 36 trials performed on 15 rats, show that BDNF microinjections in the SwCPG induced a significant dose-dependent decrease in number (F3: 44.98; P < 0.0001 Fig 1B and 2A1) and in mean intensity (F3 = 40.37; P < 0.0001; Fig 2A2) of swallows recorded during SLN stimulation. At 36 fmol (11 trials, 4 rats) and 72 fmol BDNF (10 trials, 5 rats) doses, these effects occurred during the stimulating pulse immediately following BDNF microinjection (Fig. 1B and 2A). Considering that stimulations were repeated every 30 s, it can be deduced that the latency of these BDNF inhibitory effects was under 30 s. The BDNF inhibitory effects were transient, with a significant decrease in the number and mean intensity of swallows until 240 s for 72 fmol BDNF. The decrease was maximal 60 s after BDNF microinjection, reaching 55% for the number of swallows (P < 0.0001) and 60% for the mean intensity (P < 0.0001). In comparison, at 36 fmol BDNF inhibitory effects were shorter (210 s for the number of swallows and 180 s for the mean intensity of swallows) and less powerful, with a maximal 30% decrease in the number of swallows observed 90 s after BDNF microinjection (P < 0.05) and a maximal 28% decrease in the mean intensity of swallows observed 30 s after injection (P < 0.05). In contrast, BDNF at 7.2 fmol (15 trials, 4 rats) failed to significantly alter the number and intensity of swallows, therefore this BDNF dose could be considered as subthreshold under our experimental conditions.

Since the BDNF inhibitory effect was rapid, transient and maximal during the first 90 s after BDNF microinjection, we quantified the mean number and intensity of swallows during this time course, corresponding to the first three SLN stimulations after BDNF microinjection. These results were compared to the three respective control sequences recorded before BDNF microinjection, and numerical values are summarized in table 1. At the 72 fmol BDNF dose, reductions in the 90 s mean number and intensity reached 50% (P < 0.001 and P < 0.05 respectively) when compared to control (Fig. 2B and Table 1). At the 36 fmol dose, reductions
in the 90 s mean number and intensity reached respectively 30% \((P < 0.001, \text{Fig. 2B1})\) and 27% \((P < 0.01, \text{Fig. 2B2})\) when compared to controls (Table 1).

BDNF at the 72 fmol dose inhibited the number and intensity of swallows, but without inducing any variation in cardiac frequency \((100 \pm 6.70\% \text{ for control versus } 105.91 \pm 1.79\% \text{ after BDNF injection, } P > 0.05, n = 10, \text{data not shown})\), nor in respiratory frequency \((100 \pm 7.97\% \text{ for control versus } 101.61 \pm 3.96\% \text{ after BDNF injection, } P > 0.05, n = 10, \text{data not shown})\).

**Blockade of TrkB receptors impaired the inhibitory effect of BDNF on swallowing**

When BDNF (36 fmol) was co-injected with K-252a (25 fmol), it no longer reduced the number \((P > 0.05, n = 14; \text{Fig. 3A})\) nor the intensity \((P > 0.05, n = 14, \text{Fig. 3B})\) of swallows recorded during SLN stimulation. It is worth noting that the injection of TrkB receptor inhibitor K-252a by itself had no effect on the number \((P > 0.05, n = 13; \text{Fig. 3A})\) nor on the intensity \((P > 0.05, n = 13; \text{Fig. 3B})\) of swallows triggered by SLN stimulation.

**Inhibition of swallowing : interaction between BDNF and GABAergic signalling**

In order to define the threshold level of GABA inhibiting swallowing, various doses were applied: 0.1 fmol, 0.1 pmol and 0.1 nmol. As previously shown, a marked inhibition was obtained with GABA 0.1 nmol (9) while no effect was recorded with GABA 0.1 fmol (data not shown). ANOVA analysis revealed that GABA 0.1 pmol also failed to induce a significant inhibitory effect on the number \((\text{Fig. 4A1, } F_5 = 0.445; P > 0.05, n = 10)\) and intensity \((\text{Fig. 4A2, } F_5 = 1.396; P > 0.05)\) of swallows recorded during the SLN repetitive stimulation. Thus, we considered that GABA 0.1 pmol was the subthreshold dose to induce an inhibition of swallowing under our experimental conditions. However, at effective doses, both GABA and BDNF inhibited rhythmic swallowing. In order to evaluate a putative interaction of these compounds in their inhibitory effects, GABA and BDNF were co-injected at subthreshold doses, ineffective in significantly inhibiting swallowing by themselves (i.e., GABA 0.1 pmol and BDNF 7.2 fmol). As can be seen on Fig. 4A1 and 4A2, such BDNF and GABA co-injections induced a significant decrease in the number \((\text{Fig. 4A1;} F_5 = 2.698; P < 0.05, n = 11)\) and intensity \((\text{Fig. 4A2;} F_5 = 2.663; P < 0.05)\) of swallows, indicating a synergistic effect of the two substances.

In the same manner as for BDNF inhibitory effects, we quantified the mean number and intensity of swallows during the first 90 seconds after microinjections. The results obtained were compared to the three respective control sequences recorded before each microinjection (Fig.
(P > 0.05). However, when co-injected, 7.2 fmol BDNF and 0.1 pmol GABA led to a significant decrease in the 90 s mean number (21%, P < 0.001; Fig. 4B1) and intensity (25%, P < 0.05; Fig. 4B2) of swallows.

**Blockade of BDNF inhibitory effect on swallowing by a GABA_A antagonist**

In order to further evaluate the involvement of GABAergic signalling in BDNF inhibitory effects on rhythmic swallowing induced by repetitive SLN stimulation, we first co-injected bicuculline (250 or 500 pmol), a GABA_A receptor antagonist, with BDNF at effective dose (36 fmol). As seen in figure 2B and table 2, BDNF 36 fmol alone induced a significant decrease in the 90 s mean number and intensity of swallows. Bicuculline at 250 pmol did not alter the swallowing reflex at 90 s: neither the mean number (P > 0.05, n = 8; Fig. 5A1, Table 2) nor the mean intensity (P > 0.05, n = 8; Fig. 5A2, and Table 2) was modified. However, when co-injected with 36 fmol BDNF, 250 pmol bicuculline impaired BDNF inhibitory effect on the 90 s mean number (P > 0.05, n = 15; Fig 5A1, and Table 2) and intensity (P > 0.05, n = 15; Fig. 5A2, and Table 2) of swallows. As previously shown (43), bicuculline induced a potentiation of swallowing. Indeed, an injection of bicuculline 500 pmol resulted in an increase in the 90 s mean number (60%, P < 0.01, n = 11; Fig. 5A1, and Table 2) and intensity (84%, P < 0.01, n = 11; Fig. 5A2 and Table 2) of swallows, revealing a tonic GABAergic inhibition. Interestingly, when co-injected with 36 fmol BDNF, this facilitatory effect of 500 pmol bicuculline was not reduced (P > 0.05, Fig. 5B): neither the 90 s mean number nor the intensity of swallows was changed. In consequence, when co-injections of BDNF with bicuculline were compared with control sequences recorded before any injection, the number and intensity of swallows were significantly increased (Table 2).

In order to further investigate the blockade of BDNF inhibitory effect by bicuculline, 72 fmol BDNF were injected within the SwCPG 5 min (n = 6), 15 min (n = 6) or 30 min (n = 7) after 500 pmol bicuculline microinjection (Fig. 6 and Table 2). Five minutes after bicuculline microinjection, no inhibitory effect of BDNF on the swallowing reflex was recorded: neither the 90 s mean number (P > 0.05; Fig. 6A) nor the intensity (P > 0.05; Fig. 6B) of swallows was significantly changed. Fifteen minutes after bicuculline microinjection, a slight but significant BDNF inhibitory effect was observed. Indeed, a decrease in the 90 s mean number (23%, P < 0.05; Fig. 6A) as well as in intensity (21%, P < 0.05; Fig. 6B) was recorded. However, it was necessary to wait until 30 min after bicuculline microinjection to obtain an inhibitory effect of
BDNF similar to that observed when BDNF alone was injected (number: 48% decrease, $P < 0.001$, Fig. 6A; intensity: 53% decrease, $P < 0.001$, Fig. 6B).

**Control experiment involving microinjection within the SwCPG**

Control experiments (trials on 4 different rats) indicated that microinjections of 0.9% NaCl within the SwCPG affected neither the 90 s mean number (100 ± 4.01 % before injection versus 103.23 ± 4.96 % after NaCl injection; $P > 0.05$, data not shown) nor the 90 s mean intensity of swallows (100 ± 2.1 % before injection versus 99.95 ± 2.28 % after NaCl injection; $P > 0.05$, data not shown). Moreover, in some experiments, NaCl 0.9 % was injected within the SwCPG at various times (3 - 10 min) before the injection of BDNF (72 fmol). In all cases, the inhibitory effect of BDNF on the number and intensity of swallows was unaltered (data not shown).

**SLN stimulation increased endogenous BDNF content within the DVC**

In addition, we investigated whether endogenous BDNF levels could be modified by the stimulation of laryngeal afferent fibers. Using the same SLN stimulation paradigm as the one used to study swallowing (i.e. 5s each 30s) for two hours, our results showed a significant decrease in BDNF protein content in the DVC (100 ± 6.47% for control versus 79.84 ± 3.66% for stimulated animals, $P < 0.05$). In contrast, no significant change in BDNF protein content was recorded (100 ± 3.88% for control versus 111.11 ± 6.94% for stimulated animals, $P > 0.05$) at the hypothalamic level (Fig. 7).
DISCUSSION

The present study provides the first evidence that in the adult rat, BDNF injection within the SwCPG inhibits the swallowing reflex, a fundamental motor component of food intake. In addition, our results show that the inhibitory effect of BDNF implies TrkB activation. Our data suggest that this inhibitory action is likely mediated through a GABAergic signalling system. Finally, using the same stimulation paradigm as that used to induce rhythmic swallowing, we demonstrated that the endogenous BDNF protein content within the DVC was decreased by stimulating laryngeal afferent fibers.

Methodological considerations

While triggering swallowing by a physiological stimulus such as slight touch, pressure, or water application in the oro-pharyngeal area would best reproduce physiological conditions, this approach was not appropriate to obtain reproducible data in anesthetized animals. Thus, since it is well established that stimulation of the SLN induces swallows identical to those elicited by other types of oro-pharyngeal stimulation (7, 9, 19), we used electrical stimulation of the SLN to trigger rhythmic swallowing in the anesthetized animal. Although in physiological conditions of ingestion, rats generally present individual swallows concomitant with a brief inhibition of respiratory activity, it should be noted that rhythmic swallowing with prolonged inhibition of breathing is also observed when rats ingest liquid diet or drink a large amount of liquid.

BDNF-TrkB signalling inhibited swallowing

BDNF microinjections within the SwCPG resulted in a transient inhibition of the rhythmic swallowing induced by SLN repetitive stimulation. This inhibitory effect appeared to be specific, since i) swallowing inhibition was never observed with 0.9% NaCl alone (present study, see also 9), ii) BDNF effect was dose-dependent, with 7.2 fmol as a subthreshold dose under our experimental conditions. Moreover, while the DVC is also an integrative center for autonomic cardiac and respiratory functions, the highest BDNF dose affecting swallowing had no effect on heart rate and respiratory frequency, confirming the role of BDNF as specific to ingestive functions rather than other autonomic functions, at the level of the SwCPG.

The specificity of BDNF effects was confirmed by the absence of inhibition of swallowing by this neurotrophin when co-injected by K-252a, a permeant membrane antagonist of TrkB receptors (24, 38). BDNF binds to two structurally unrelated plasma membrane receptor types, the low-affinity p75 neurotrophin receptor and the high-affinity TrkB receptor that
contains an intracellular tyrosine kinase catalytic domain (31, 39). Both receptors can act independently but also interact with each other (5). Interestingly, it has been shown that TrkB receptors are highly expressed within the DVC, not only on the afferent fibers but also on the efferent pathway, since neurons of the DMNX are also TrkB positive (45). Our results strongly suggest that BDNF inhibition of swallowing is specifically mediated by TrkB receptor signalling.

_BDNF interacted with GABAergic signalling to inhibit swallowing_

The SwCPG is located in the NTS, which is particularly rich in GABAergic interneurons (18). Moreover, GABA has been shown to act as an inhibitory mediator in swallowing (15, 43). In a previous study, we showed that a microinjection of GABA within the NTS induces a rapid, transient and dose-dependant inhibition of rhythmic swallowing (9). Conversely, here we show that bicuculline microinjection within the SwCPG induces a significant increase in the number and intensity of swallows. Such a facilitating effect of bicuculline on swallowing has been previously reported: bicuculline application into the fourth ventricle and microinjection into NTS induce long-lasting facilitation of swallowing (6, 43). These data suggest that GABAergic signalling plays a major role in swallowing as it exerts a tonic inhibition on this motor component of food intake. Since GABA-induced inhibitory effects (9) presented properties similar to those obtained in the present study after exogenous BDNF microinjection within the SwCPG, we tested the interaction between BDNF and GABA. We show here that co-injection of BDNF and GABA in the SwCPG at subthreshold doses induces a significant inhibition of swallowing. This result indicates that a synergistic interaction between BDNF and GABA inhibits swallowing. Therefore, the BDNF inhibition of swallowing may involve GABAergic signalling modulation, although BDNF and GABA effects on SwCPG neurons may involve two separate pathways. Since we showed that BDNF inhibitory effects were impaired when GABA receptors were blocked by bicuculline, our results support the hypothesis that GABAergic neurotransmission is an important downstream effector through which BDNF inhibits swallowing.

The potentiation of GABAergic signalling by BDNF has already been reported. This potentiation could result either from a presynaptic effect of BDNF inducing an increase in GABA release, or from a postsynaptic effect of BDNF inducing an increased expression of GABA receptors at the cell surface, or by some modifications of their properties. Thus, in the isolated dorsal horn (33) or in preparations of mammalian nerve terminals (20), BDNF facilitates the release of GABA, which in turn causes inhibition of transmission. However, in
hippocampal CA1 pyramidal neurons, BDNF rapidly and reversibly potentiates postsynaptic 
GABA_A receptor-mediated currents. In the presence of BDNF, this GABA_A response is 
potentiated through the activation of postsynaptic TrkB receptor (28). Moreover, in the rat visual 
cortex, BDNF induces a rapid increase in the total number of cell surface functional receptors 
(29). In the swallowing context, our results do not allow us to elucidate whether BDNF 
inhibitory effects are mediated by an increase in GABAergic secretion by interneurons or by an 
increased GABA receptivity on SwCPG neurons. Further studies at cell level would be required 
for a detailed breakdown of the mechanism of this interaction. Nevertheless, we have 
demonstrated the ability of BDNF to act as a fast modulator of synaptic activity in the SwCPG, 
inducing a potentiation of the GABAergic system that in turn results in an inhibition of 
swallowing.

**SLN stimulation induced a decrease in BDNF protein content within the DVC**

To further examine the involvement of BDNF in swallowing, we evaluated the effect of 
SLN stimulation on BDNF protein content within the DVC. For the present experiment we 
applied the same SLN stimulation paradigm as that used to study swallowing, i.e. 5s of efficient 
SLN stimulation every 30s. As a consequence, during the two-hour experiment, the SLN was 
effectively stimulated only for twenty minutes, and in an intermittent manner. In adult rats, the 
meal lasts about 15-20 minutes (46) Moreover, a previous study showed that, in physiological 
conditions, the laryngeal mechanoreceptor afferent fibers of the SLN discharge at frequencies 
between 10 and 20 Hz (17, 27), which is of the same order as the frequency used in our 
stimulation paradigm. Thus, the twenty-minute effective stimulation used in the present 
experiment might be compared with afferent activities triggered during a meal.

Our results showed that a two-hour intermittent SLN stimulation induced a decrease in 
endogenous BDNF protein content within the DVC, while the hypothalamus content was not 
affected. Although the swallowing reflex does not involve the hypothalamus, this structure was 
taken as a reference for the central nervous regulation of feeding. Moreover, BDNF also being 
expressed in the hypothalamus, the content of both structures could be compared. It should be 
noted that SLN intermittent stimulation induced repetitive swallowing with a concomitant 
blockade of respiratory activity. Thus, the BDNF protein content decrease observed after SLN 
stimulation could be the result of either the swallowing induced by SLN stimulation, the apnea 
obtained during SLN stimulation or both phenomena. Since we demonstrated that exogenous 
BDNF injection within the DVC inhibited swallowing without any modification in respiratory
frequency, we favored the hypothesis that modulation of BDNF obtained is a consequence of inducing swallowing.

Another recent study in the rat reported variations in BDNF expression both in the hippocampus and the cerebral cortex after vagus nerve stimulation (10). Variations were also found under more physiological conditions. For example, it was shown that whisker stimulation results in an increase in BDNF mRNA expression in the barrel cortex of adult mice (37) and rats (30). Visual deprivation (by dark rearing) results in decreased levels of BDNF mRNA in the visual cortex, and subsequent exposure to light re-establishes normal levels (3). After hindpaw sensory restriction, BDNF mRNA relative levels increase in the rat somatosensory cortex (8). In mice, exposure to music increases the BDNF level in the hypothalamus (1). Thus, the regulation of this neurotrophin expression is dependent on sensory activation. Our findings strongly suggest that BDNF modulations in the DVC may also be induced by physiological processes during which the SLN is highly activated, for example when food travels from the mouth to the gut.

BDNF protein and mRNA, as well as TrkB protein and mRNA, are found throughout the DVC, with very heavily labeled fibers and cells in the NTS (4, 45). Moreover, the protein BDNF has been shown to be actively transported along the axon, both in an anterograde and retrograde manner (for review, see 35). Thus, the modulation of endogenous BDNF protein content within the DVC could be the result of i) local modifications in neosynthesis at the DVC level, ii) modification of BDNF synthesis and/or axonal transport by neurons localized outside the DVC and sending projections to the DVC. It seems unlikely that the observed variations in BDNF content could originate from SLN or vagal afferent fibers. Indeed, there is no available data supporting the view that SLN afferent neurons express BDNF mRNA or protein, and vagal afferent fibers do not transport BDNF in an anterograde manner but only in a retrograde manner in adult rats (16). High quantities of BDNF are also expressed within viscera (26). Thus, local neosynthesis modulation within the DVC, or BDNF transport modifications from vagal efferents or superior centres such as the hypothalamus or the parabrachial nucleus (PBN), which express BDNF (4, 44) and send projections to the NTS (18), could be involved in the modulation of BDNF protein content observed at the DVC level.

**Perspectives and significance**

The major finding of our study is that BDNF/TrkB signalling inhibits swallowing via a GABA\(_A\)-dependent mechanism in the adult rat. Previously we demonstrated that BDNF is an anorexigenic factor within the DVC of the adult rat (2). However, the targets of anorexigenic (or
orexigenic) effects are not often clearly identified. Here, the results strongly suggest that swallowing inhibition can represent one of the outputs by which BDNF alters the ingestive pattern (Fig. 8). In addition to the inhibition of swallowing by BDNF, in the present study we also show that BDNF protein content within the DVC decreases after SLN stimulation. These results can be transferred to the larger physiological context of feeding control. The eating sequence involves both positive and negative feedback. The oro-motor events related to mastication and swallowing stimulate mechanoreceptors located in the upper gastrointestinal tract, thereby providing a positive feedback which allows eating to continue (25). Conversely, negative feedback is provided by the integration of signals related to satiety, such as CCK, that are emitted when the ingested food enters the lower gastrointestinal tract, and allow the eating sequence to stop. We previously showed that BDNF protein content decreases after a 48-h fast and increases after re-feeding or after CCK or leptin treatment (2). All these data suggest that BDNF could exert an anorexigenic tonus within the DVC where an increase in BDNF protein content could be a molecular basis of the integration of negative feedback induced by satiety signals. Thus, the decrease in BDNF protein content observed after SLN stimulation might result in a decrease in this anorexigenic tonus, allowing the maintenance of the eating sequence. In such a hypothesis, laryngeal afferent fiber activation could be identified as a positive feedback for the eating sequence. Interestingly, it has been suggested that the hyperphagia of BDNF+/- obese mice implicates an enhancement of positive feedback activated by the sensory receptors that supply oro-pharyngeal structures (11). Conversely, an increase in BDNF protein content depending on lower gastrointestinal signals could explain the swallowing disfacilitation observed at the end of the meal in decerebrate rats although food is still present in the mouth (12-14). Finally, BDNF could be considered as a common integrator for both positive and negative feedback controlling food intake behavior at the DVC level (Fig. 8).
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FIGURE LEGENDS

Fig. 1: Polygraphic recordings during the experiment.
A: Representative view of parameters recorded in the rat. Stim, stimulation of the superior laryngeal nerve (SLN) at 20 pulses/s during 5 s (pulses parameters 1.5 V and 0.2 ms); EMG, electromyogram from sublingual muscles. Note the rhythmic swallowing triggered by SLN stimulation; ECG, electrocardiogram; Resp, respiration. Note the respiration blockade during the SLN stimulation triggering rhythmic swallowing. ÊEMG, electromyogram envelope signal normalized. B: BDNF microinjection (36 fmol) within the medullary NTS inhibited rhythmic swallowing triggered by SLN stimulation. Note that the inhibition of rhythmic swallowing was rapid (30 s after BDNF injection) and transient (recovery after 210 s). Stimulation parameters were 3 V, 1.5 ms, 25 Hz during 5 s.

Fig. 2: BDNF microinjections within the SwCPG inhibited rhythmic swallowing triggered by SLN stimulation.
A: Time course of the effects of various doses (7.2 fmol, n = 15; 36 fmol, n = 11 or 72 fmol, n = 10) of brain-derived neurotrophic factor (BDNF) microinjections within the medullary NTS on (A1) the number and (A2) mean intensity of swallows triggered by SLN stimulation. Note time course variations according to the doses. Data represent the time after BDNF microinjection and until recovery. B: Graphic representation of the 90 s maximal intensity dose-dependent BDNF-induced inhibition of swallowing. Note that the 90 s mean number (B1) and intensity (B2) of swallows significantly decreased with increasing doses. Values are mean ± SEM normalized to the respective responses recorded before BDNF microinjection (Ctrl), * P<0.05, ** P<0.001; *** P<0.0001.

Fig. 3: Involvement of TrkB receptor in BDNF-induced swallowing inhibition.
Time course of K-252a injections (25 fmol; n = 13) compared with K-252a (25 fmol) and BDNF (36 fmol) co-injections (n = 14) within the SwCPG on the number (A), and intensity (B) of swallows elicited by SLN stimulation. Note that no modification was recorded. Values are mean ± SEM normalized to the respective responses recorded before injection (Ctrl).

Fig. 4: Synergistic interaction between BDNF and GABA to inhibit rhythmic swallowing.
A: Time course of the effects of injections of BDNF (7.2 fmol, n = 15) and GABA (0.1 nmol, n = 10) alone compared with co-injections of 7.2 fmol BDNF and 0.1 nmol GABA (n = 11) within
the SwCPG on the number (A1) and intensity (A2) of swallows. B: Graphic representation of the number (B1) and intensity (B2) of swallows triggered by SLN stimulation during the first 90 s after injections of BDNF (7.2 fmol, n = 15) and GABA (0.1 nmol, n = 10) alone and co-injections of 7.2 fmol BDNF and 0.1 nmol GABA (n = 11). Note that BDNF and GABA alone did not change the swallowing parameters while a co-injection elicited a significant decrease in the number (A1 and B1) and intensity (A2 and B2) of swallows. Values are means ± SEM normalized to the respective responses recorded just before injection, ns: non-significant, * P < 0.05, ** P < 0.001, *** P < 0.0001.

Fig. 5: BDNF inhibitory effects on rhythmic swallowing is impaired by bicuculline
Effects of bicuculline alone (250 pmol, n = 8; or 500 pmol, n = 11) or co-injected with BDNF 36 fmol within the SwCPG on 90 s mean number (A) and intensity (B) of swallows triggered by SLN stimulation. Bicuculline 250 pmol had no effect on the mean number and intensity of swallows, while bicuculline 500 pmol induced a significant increase on the number and intensity of swallows. Moreover, bicuculline blocked BDNF inhibitory effects; neither the 90 s mean number nor intensity of swallows were reduced when BDNF was co-injected with bicuculline 250 pmol (n = 15) or 500 pmol (n = 12). Values are means ± SEM normalized to the respective responses recorded just before injection, ns: non-significant.

Fig. 6: Bicuculline blockade of BDNF inhibitory effects on swallowing was reversible.
BDNF microinjections were performed 5 min (n = 6), 15 min (n = 6) or 30 min (n = 7) after bicuculline 500 pmol. BDNF injected 5 min after bicuculline had no significant effect on swallowing. When BDNF was injected 15 min later, the 90 s mean number (A) and intensity (B) of swallows were significantly lowered. After 30 min, BDNF inhibitory effects were greater, number (A) and intensity (B) of swallows triggered by SLN stimulation were significantly reduced. Values are means ± SEM normalized to the respective responses recorded just before injection, *** P<0.0001.

Fig. 7: Comparison of endogenous BDNF protein content within the DVC and hypothalamus.
After long trains of SLN stimulation (1.5 V; 0.2 ms; 30 Hz ; 5 s every 30 s for 2 hours; n = 5) a significant decrease in BDNF protein content was measured within the DVC but not within the hypothalamus of the same rats. Each column represents the mean relative BDNF protein content measured after two hours of rhythmic swallowing induced by SLN stimulation compared to
sham control group (n = 5). Values are mean ± SEM normalized to the respective sham control values. ns: non-significant, * P<0.05.

Fig. 8: Schematic view of BDNF acting in the DVC as a common integrator for both positive and negative feedbacks controlling the ingestive sequence.
BDNF: brain-derived neurotrophic factor, DVC: dorsal vagal complex, GABA: γ-aminobutyric acid, GI tract: gastrointestinal tract, SLN: superior laryngeal nerve, SwCPG: swallowing central pattern generator, X: vagus nerve. (see text for more details)

Table 1: Changes in number and intensity of swallows triggered by SLN stimulation recorded during the first 90 s after BDNF injections at different doses.
Values are means ± SEM normalized to the respective responses recorded 90 s before BDNF injection, ns: non-significant, * P < 0.05, ** P < 0.001, *** P < 0.0001.

Table 2: Changes in number and intensity of swallows triggered by SLN stimulation recorded during the first 90 s after injections of various drugs.
Values are means ± SEM normalized to the respective responses recorded 90 s before BDNF injection, ns: non-significant, * P < 0.05, ** P < 0.001, *** P < 0.0001.
A

Stim

EMG

ECG

Resp

∫EMG

Stim

B

Control

30 s

150 s

210 s

Stim
A

Number (%)

Time (s)

B

Intensity (%)

Time (s)

K-252a
K-252a + BDNF 36 fmol

Legend:

- ■ K-252a
- □ K-252a + BDNF 36 fmol
A1  B1

A2  B2

BDNF 7.2 fmol
GABA 0.1 nmol
BDNF 7.2 fmol + GABA 0.1 nmol

90s Mean Number (%)

90s Mean Intensity (%)

Ctrl 30s 60s 90s 120s 150s

Time (s)

BDNF 7.2 fmol
GABA 0.1 nmol
BDNF 7.2 fmol + GABA 0.1 nmol

ns

**

***
A

90 s Mean Number (%)

B

90 s Mean Intensity (%)

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<th>500 pmol</th>
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</table>
Bicuculline (500 pmol) +
BDNF (72 fmol) +

A  90 s Mean Number (%)

B  90 s Mean Intensity (%)

5 min 15 min 30 min

*** *** **
BDNF
SwCPG - GABA
Dorsal Vagal Complex
Positive feedback
Negative feedback
Upper GI tract
SLN (X), Blood
Lower GI tract
Maintenance of ingestion
End of ingestion
Anorexigenic tonus
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### A

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