Contribution of the vagus nerve and lamina terminalis to brain activation induced by refeeding

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Abstract
Following refeeding, c-fos expression is induced in a particular set of brain regions that include the nucleus of the solitary tract (NTS), parabrachial nucleus (PB), central amygdala (CeA), paraventricular hypothalamic nucleus (PVH), supraoptic nucleus (SON) and the circumventricular organs. Within the PVH, the expression is particularly intense in the magnocellular division of the nucleus and it is as yet not clear how this activation occurs. The respective contribution of the vagus afferents and lamina terminalis, which conveys signals entering the brain through the forebrain circumventricular organs, has been investigated in rats subjected to a unilateral cervical vagotomy (UCV) or a unilateral lesion of the fibres running within the lamina terminalis (ULT) and projecting to the neuroendocrine hypothalamus. UCV significantly decreased postprandial c-fos expression in the NTS, PB, CeA and parvocellular division of the PVH. In contrast, ULT impaired postprandial activation of the magnocellular neurons in the PVH and SON. The present study also characterized the types of neurons activated in the PVH and SON during refeeding. In the magnocellular regions, arginine-vasopressin (AVP) neurons were activated upon refeeding whereas there was no apparent induction of Fos expression in oxytocin cells. In the paraventricular PVH, postprandial Fos was induced only in 30% of the corticotrophin-releasing factor (CRF) and AVP neurons. The results of the present study suggest that the postprandial activation of the brain requires the integrity of both the vagal- and lamina terminalis-associated pathways.

Introduction
Feeding activates a selective set of brain regions related to gustatory/viscerosensory processing, fluid balance, and parasympathetic control, which include the nucleus of the solitary tract (NTS), parabrachial nucleus (PB), central nucleus of amygdala (CeA), arcuate (Arc) and paraventricular (PVH) hypothalamic nuclei, supraoptic nucleus (SON) and circumventricular organs (Fraser & Davison, 1993; Fraser et al., 1995; Naimi et al., 1997; Timofeeva et al., 2002; Pecoraro & Dallman, 2005). Following eating, these regions express the immediate early gene c-fos, a widely used marker of functional neuronal activations (Sagar et al., 1988; Sawchenko et al., 2000). Expression of c-fos has been found to be particularly reliable to measure brain activations in response to stressful conditions (Sawchenko et al., 1996; Herman, 1999) and feeding (Naimi et al., 1997; Timofeeva et al., 2002; Pecoraro & Dallman, 2005).

There is evidence that food ingestion may in some circumstances lead to central activations initiated from the cortex (Pecoraro & Dallman, 2005). However, food-related signals reach neuronal pathways in the central nervous system (CNS) mainly by travelling through either vagal afferents or blood circulation. Vagal afferents enter the NTS, which constitutes the first central relay for vagally mediated signals emerging from gastric distension and gastrointestinal peptides, such as cholecystokinin (CCK), which is certainly one of the strongest inducers of Fos expression in the NTS as well as in its midbrain and forebrain relays (Monnikes et al., 1997; Schreihofer et al., 1997; Berthoud et al., 2001; Glatzle et al., 2001; Vrang et al., 2003). Interruption of the vagal sensory pathway produced by subdiaphragmatic vagotomy or cytotoxic lesion of the noradrenergic NTS neurons significantly attenuated Fos induction in selective brain regions after intragastric salt loading or systemic CCK administration (Carlson & Osborn, 1998; Starbuck et al., 2002; Rinaman, 2003). On the other hand, circulation, which readily accesses circumventricular organs (CVOs), conveys signals associated with circulating gut hormones. CVOs, which include the area postrema, the subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT), are devoid of the blood–brain barrier and express numerous receptors of hormones capable of influencing the brain (la Fleur et al., 2003; Yamamoto et al., 2003; Cottrell & Ferguson, 2004; Woods, 2004). The role of the CVOs, in particular those lining the lamina terminalis (the SFO and OVLT), on the response to signals led to by food ingestion, is still largely unknown.

The present study was designed to investigate the role of the cellular groups of the lamina terminalis and the vagus afferents in the central activation led to by the ingestion of a meal after 24 h of fasting. Refeeding after a period of fasting has proven to be a reliable model to investigate feeding-induced brain activations (Naimi et al., 1997; Timofeeva et al., 2002) as it allows for a clear illustration of the structures expressing c-fos in response to food. Rats were subjected to either a unilateral cervical vagotomy (UCV) or a unilateral lesion of the fibres running from the cellular groups of the lamina terminalis to the neuroendocrine hypothalamus (ULT — unilateral lamina terminalis cuts). In rats with UCV, the left vagus nerve, which transfers the

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majority of gastrointestinal signals to the central nervous system (Rinaman & Schwartz, 2004), was severed, whereas the right vagus nerve was preserved to maintain normal cardiac activity.

Materials and methods

Animals and feeding

Male Wistar rats weighing 260–300 g were purchased from the Canadian Breeding Laboratories (St-Constant, Canada). All rats were cared for and handled according to the Canadian Guide for the Care and Use of Laboratory Animals, and the present protocol was approved by our institutional animal care committee. The animals were housed individually in wire-bottom cages suspended above absorbent paper, and, unless otherwise specified, fed ad libitum with a stock diet (Agway Prolab, Rat/Mouse/Hamster 1000 Formula). They were subjected to a 12-h light : 12-h dark cycle and kept under an ambient temperature of 23 ± 1 °C. Rats were killed at the beginning of a light phase either in an ad libitum fed state or after 1 h of refeeding following 24 h of food deprivation.

Nerve transections

All surgeries were carried out under ketamine–xylazine anaesthesia (3 mL/kg of a mixture containing 20 mg/mL of ketamine and 2.5 mg/mL of xylazine). UCV (n = 6) was produced by transecting the left cervical vagal nerve trunk distal to the emergence of the superior laryngeal nerve. The neck skin was thereafter closed by interrupted silk sutures and the animals were allowed to recover for 7 days. The same procedures were performed on rats (n = 6) with sham cervical vagotomy with the exception that the vagus nerve was not severed.

Forebrain unilateral transections (n = 6) designed to cut the fibres running from the lamina terminalis to the hypothalamus were produced stereotaxically in a coronal plane using a blade of 2-mm width. Cuts were positioned perpendicular to the midline and caudal to the OVLT (bregma −0.26). Only animals (n = 4) bearing complete cuts that extended to the midline and reached the base of the brain, aimed to sever completely the projections of the lamina terminalis to the hypothalamus (Miselis et al., 1979; Saper & Levisohn, 1983; Sawchenko & Swanson, 1983), were included in the analyses. Animals with cuts of similar size and orientation but positioned rostrally to the OVLT (bregma −0.26) were excluded. No animals (n = 4) bearing complete cuts were included in the analyses. Animals with cuts of similar size and orientation but positioned rostrally to the OVLT were used as controls (n = 4).

Anterograde tracing

The anterograde tracer biotinylated dextran (BDA), high-molecular weight (2% BDA 10 kDa; Molecular Probes, Eugene, OR) was iontophoretically infused using positive current pulses (1 μA, 15 min, 2-s pulses) in the medial part of the left NTS (n = 7, coordinates 13.24 mm caudal and 1.1 mm lateral to the bregma; 4.9 mm ventral from the brain surface), and in the left part of the ventral region of the lamina terminalis (n = 8, coordinates 0.2 mm rostral and 0.1 lateral to the bregma; 8.4 mm ventral from the brain surface). One week following the surgeries or the infusion of the tracer, rats were killed after 1 h of refeeding following 24 h of food deprivation.

Brain preparation

Rats were anaesthetized with 3 mL/kg of a mixture containing 20 mg/mL of ketamine and 2.5 mg/mL of xylazine. Without delay, they were intracardially perfused with 200 mL of ice-cold isotonic saline followed by 500 mL of a paraformaldehyde (4%) solution. The brains were removed at the end of perfusion and kept in paraformaldehyde for an additional period of 7 days. They were then transferred to a solution containing paraformaldehyde (4%) and sucrose (10%) before being cut 12 h later using a sliding microtome (Histoslide 2000, Reichert-Jung, Heidelberg, Germany). Brain sections were taken from the olfactory bulb to the brainstem. Thirty-μm-thick sections of brains were collected and stored at −30 °C in a cold cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%).

In situ hybridization histochemistry

In situ hybridization histochemistry was used to localize c-fos mRNA on tissue sections taken from the entire brain. The used protocol for in situ hybridization was largely adapted from the technique described by Simmons et al. (1989). Briefly, the brain sections were mounted onto poly L-lysine coated slides and allowed to desicate overnight under vacuum. They were then successively fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37 °C with proteinase K (10 μg/mL in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After vacuum drying for at least 2 h, 90 μL of the hybridization mixture, which contains an antisense 35S labelled cRNA probe (10 8 cpm/μL), were spotted on each slide. The slides were sealed under a coverslip and incubated overnight at 60 °C in a slide warmer. The next day, coverslips were removed and the slides rinsed four times with 4× SSC (0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7.0), digested for 30 min at 37 °C with RNaseA (20 μg/mL in 10 mM Tris–500 mM NaCl containing 1 mM EDTA), washed in descending concentrations of SSC (2×, 10 min; 1×, 5 min; 0.5×, 5 min; 0.1×, 30 min at 60 °C) and dehydrated through graded concentrations of alcohol. After a 2 h-period of vacuum drying, the slides were exposed on an X-ray film (Eastman Kodak, Rochester, NY) for 24 h. Finally, tissues were rinsed in running distilled water for 1–2 h, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

Antisense 35S-labelled riboprobes

The c-fos cRNA probe was generated from the EcoRI fragment of rat c-fos cDNA (Dr I. Verma, The Salk Institute, La Jolla, CA, USA), subcloned into pBluescript SK-1 plasmid (Stratagene, La Jolla, CA). The corticotropin-releasing factor (CRF) cRNA probe was generated from the EcoRI fragment of a rat CRF cDNA (Dr K. Mayo, North-western University, Evanston, IL), subcloned into pGem-4 plasmid (Stratagene, La Jolla, CA). Radioactive riboprobes were synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, 10 mM dithiothreitol, 6 mM MgCl2, 40 mM Tris (pH 7.9), 0.2 mM ATP/GTP/CTP, α-35S-UTP, 40 U RNasin (Promega, Madison, WI), and 20 U of T7 or T3 RNA polymerase for, respectively, antisense and sense probes of c-fos RNA; and SP6 or T7 RNA polymerase for, respectively, antisense and sense probes of CRF RNA for 60 min at 37 °C. The DNA templates were treated.
with 100 µL of DNAse solution (1 µL DNAse, 5 µL of 5 mg/mL tRNA, 94 µL of 10 mM Tris/10 mM MgCl₂). The preparation of the riboprobes was accomplished the phenol–chloroform extraction and ammonium acetate precipitation. The specificity of the probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

**Double-labelling procedures**

Immunohistochemical detection of Fos, the protein encoded by the oncogene c-fos, was combined with detection of CRF mRNA by *in situ* hybridization to determine whether CRF cells were activated during refeeding. Brain sections were first processed for immunohistochemical detection of Fos using a conventional avidin-biotin-immunoperoxidase method. Briefly, brain slices were washed in sterile 0.05 M potassium phosphate-buffered saline (KPBS, pH 7.2) that was treated with diethyl pyrocarbonate (DEPC) water. They were then incubated for 24 h at 4 °C with a Fos antibody (rabbit polyclonal IgG, Oncogene Science, NY). The Fos antibody was used at a 1 : 50 000 dilution in KPBS (50 mM) with heparin (0.25%), Triton X-100 (0.4%) and bovine serum albumin (2%). Twenty-four hours following incubation at 4 °C with the first antibody, the brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS, Triton X-100, heparin, and biotinylated goat antirabbit IgG (1 : 1500 dilution; Vector Laboratories, CA) for 90 min. Sections were then rinsed with KPBS and incubated at room temperature for 60 min with an avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, CA). After several rinses in KPBS, the brain slices were allowed to react in a mixture containing KPBS, 0.05% DAB, and 1% hydrogen peroxide (brown reaction product).

To locate the fibres projecting from the NTS or the ventral region of the lamina terminalis associated with the Fos protein, brain sections were first processed for immunohistochemical detection of BDA using the ABC complex (Vector Laboratories, Burlingame, CA) and nickel-cobalt-DAB (black reaction product). Thereafter, tissues were rinsed in KPBS and processed for immunohistochemical detection of Fos protein that was performed through the incubation of the brain sections with the primary antibody as described above and a peroxidase-labelled secondary antibody (goat antirabbit IgG, Chemicon), revealed with the chromagen DAB as a substrate (brown reaction product).

**Quantitative analysis of the hybridization signals**

The hybridization signals revealed on NTB2 dipped nuclear emulsion slides were analysed and quantified under a light microscope (Olympus, BX50) equipped with a black and white video camera (Sony, XC-77) coupled to a Macintosh computer (Power PC 7100/66) using Image software (version 1.57 non-FPU, Wayne Rasband, NIH, Bethesda, MD). The intensity of the hybridization signal was measured under dark-field illumination at a magnification of 25×. To avoid the saturation of the hybridization signal, a calibration density profile plot was created. The luminosity of the system was then corrected so that, for the strongest hybridization signals, the reading optical density (OD) did not exceed half of the maximal pixel value, and this luminosity was conserved throughout the analysis of the entire series. The paraventricular and magnocellular PVH (bregma from −1.80 to −2.00), SON (bregma from −1.10 to −1.40), dorsomedial hypothalamic nucleus (DMH; bregma from −3.14 to −3.30), CeA (bregma from −2.12 to −2.80), Arc (bregma from −2.12 to −3.5), Kölliker-Fuse nucleus (KF) and parabrachial nuclei (PB); (bregma from −9.16 to −9.68), NTS, divided in its longitudinal axis to the rostral part (NTSr, at the vicinity of the spinal trigeminal nucleus, bregma from −11.00 to −12.95), medial part (NTSm, at the level at which the NTS touches the fourth ventricle, bregma from −13.00 to −13.60), caudal part (NTSc, caudal to the obex, bregma from −13.68 to −14.60) were outlined and the measurements of the OD of the hybridization signal were performed separately on each side of the brain on the 4–6 sections for each animals assigned to each treatment. When no hybridization signal was visible under darkfield illumination, the brain structures of interest were outlined under brightfield illumination and then subjected to densitometric analysis under darkfield illumination. The OD for each specific region was corrected for the average background signal, which was determined by sampling unlabelled areas outside of the areas of interest. The brain sections from the different groups of rats were matched for rostrocaudal levels as closely as possible. The mean of the measurements for individual animals were used to determine the mean ± SEM for each group.

**Statistical analysis**

One-way analysis of variance (ANOVA) and two-tailed Student’s *t*-test were used to examine the effects of the various treatments on the dependent variables measured in this study. *A posteriori* comparisons between groups were realized using the Fisher’s Protected Least Squares Difference.
Results

To identify the central circuitries involved in the transmission of feeding-associated signals to the neuroendocrine hypothalamus, we have studied the postprandial induction of expression of the immediate early gene c-fos, which has proven to be a sensitive marker for the functional neuronal activation by a variety of stimuli (Morgan & Curran, 1991). Animals \((n = 6)\) killed after one hour of refeeding following 24 h of food deprivation demonstrated a strong induction of c-fos expression \((P < 0.001,\) compared to \(ad \) libitum fed rats, \(n = 4)\) in specific brain regions that included the NTS, lateral and medial PB (respectively, PBl and PBm), KF, CeA, paraventricular thalamic nucleus (PVT), DMH, Arc, SON, OVLT, magnocellular PVH and parvocellular PVH (to a lesser extent), therefore replicating the recently reported pattern of central activation led to by refeeding (Timofeeva et al., 2002). Central regions activated in response to refeeding were exactly the same that expressed Fos protein immediately after a session of feeding (Racotta et al., 1995; Naimi et al., 1997). In the \(ad \) libitum fed group, which was killed at the beginning of the diurnal period, c-fos expression was at near-background levels in agreement with previous reports. The effect of 24 h of food deprivation was not assessed in the present study as we recently reported no marked c-fos expression in control rats, which were food-deprived for 24 h (Timofeeva & Richard, 2001; Timofeeva et al., 2002).

Combination of Fos-immunoreactivity (ir) with the detection of CRF transcripts, AVP-ir or oxytocin-ir revealed that the postprandial Fos-ir in the magnocellular PVH and SON was mainly localized in AVP neurons (Figs 1 and 2), as more than half of AVP magnocellular neurons coexpressed Fos \((55.7 \pm 9.3\%\) and \(78.0 \pm 7.8\%\) of AVP neurons, in the magnocellular PVH and SON, respectively, were positive for Fos-ir). Expression of Fos in the oxytocin magnocellular neurons in refed animals was considerably low \((5.3 \pm 1.6\%\) and \(7.9 \pm 1.8\%\) of oxytocin neurons, in the magnocellular PVH and SON, respectively, were positive to Fos-ir; Figs 1 and 2).

In the parvocellular PVH, only \(28.1 \pm 9.0\%\) of CRF neurons coexpressed Fos after one hour of refeeding (Figs 1 and 2). CRF mRNA/Fos-ir double-labelled neurons were located mostly in the ventral and dorsal subdivisions of the paravocellular PVH, whereas in the medial subdivision the number of double-labelled cells was relatively low. Fos immunoreactivity was detected in \(36.7 \pm 13.7\%\) and \(4.3 \pm 1.04\%\) of, respectively, AVP and oxytocin neurons in the paravocellular PVH in refed animals (Fig. 1). Interestingly, the fact that only \(55.0 \pm 19.5\%\) of Fos-ir cells in the paravocellular PVH were CRF, AVP and oxytocin positive neurons, suggests that refeeding induced Fos expression in other types of parvocellular neurons in addition to the above-mentioned neurosecretory cells.

UCV significantly decreased the expression of c-fos mRNA after one hour of refeeding in nuclei ipsilateral to the vagus transection,

![Graph](image.png)

**Fig. 1.** Induction of Fos expression in response to refeeding in the CRF, AVP and OT neurons in the SON and parvocellular (PVHp) and magnocellular (PVHm) parts of the paraventricular hypothalamic nucleus. Values are mean numbers of cells (± SEM) per 30-μm section expressing CRF mRNA, or immunopositive for AVP and OT (white bars), positive for Fos (hatched bars), or coexpressing Fos and CRF, AVP or OT (black bars). White and black bars were superimposed to emphasize the proportion of neurohormonal cells expressing Fos protein during refeeding.

![Graph](image.png)

**Fig. 2.** Postprandial Fos expression in the CRF, AVP and OT neurons in the PVH and SON (left panels are the higher magnification of the areas depicted by rectangles on the right panels). (A) CRF mRNA hybridization signal (silver grains) and Fos-immunoreactivity (brown nuclei) in the parvocellular and magnocellular PVH (PVHp and PVHm). High-magnification photomicrograph (F) demonstrates the PVHp neurons expressing Fos protein, but not CRF mRNA (black arrowheads), the cells positive to CRF mRNA hybridization signal, but not to Fos immunoreactivity (white arrowheads), and the neurons coexpressing CRF mRNA and Fos protein (black arrow). (B) AVP/Fos cells in the PVH. (G) High-magnification photomicrograph depicts Fos expression (black nuclei) in the AVP-immunoreactive (ir) neurons (brown-stained cells) in the PVHm (some double-staining neurons are indicated by black arrows). (C) AVP/Fos double-immunostaining in the SON. The Fos protein (black nuclei) is expressed in the majority of AVP-ir neurons (brown-stained cells) in the SON (H; some double-staining neurons are indicated by black arrows). (D) OT-ir and Fos-ir cells in the PVH. (E) OT-ir and Fos-ir cells in the SON. Note on the high-magnification photomicrographs that in the PVHm (I) and in the SON (J) the cells expressing OT (brown staining, indicated by white arrowheads) and postprandial Fos (black nuclei, indicated by black arrowheads) represent the separate clusters of neurons. Scale bars, 100 μm (right panels) and 20 μm (left panels).
which included the caudal and medial NTS, KF and PBl (Figs 3 and 4). UCV did not affect postprandial c-fos expression in the rostral NTS and PBm (Figs 3 and 4). In the forebrain, the decrease in postprandial c-fos expression in the ipsilateral hemisphere was detected in the CeA and parvocellular PVH (Figs 3 and 4). In the magnocellular PVH, SON, DMH, PVT and Arc, c-fos mRNA expression in response to refeeding was not apparently affected by UCV (Figs 3 and 4).
As UCV was ineffective in preventing the postprandial activation of the magnocellular neurons, a series of experiments were designed to test the influence of the descending pathway from the cell groups of the lamina terminalis to the PVH and SON. Unilateral transections caudal to the OVLT were stereotaxically performed (Fig. 5) and aimed at disrupting fibres running from the OVLT and SFO to the PVH and

![Graphs showing optical density (OD) of the hybridization signal for c-fos mRNA in ad libitum fed intact rats, and in animals refed for one hour after 24 h of food deprivation following unilateral cervical vagotomy or sham-unilateral cervical vagotomy. Measurements were made in the parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVHp and PVHm), SON, CeA, DMH, Arc, medial and lateral parabrachial nuclei (respectively PBm and PBl), KF, rostral, medial and caudal parts of the nucleus of the solitary tract (respectively NTSr, NTSm, NTSc). * Significantly (P<0.05) different from the contralateral side in the same operation and the same nutrition condition. AI, ad libitum, ipsilateral; AC, ad libitum, contralateral; SI, sham-operated, ipsilateral; SC, sham-operated, contralateral; VI, left cervical vagotomy, ipsilateral; VC, left cervical vagotomy, contralateral.

Fig. 3. Optical density (OD) of the hybridization signal for c-fos mRNA in ad libitum fed intact rats, and in animals refed for one hour after 24 h of food deprivation following unilateral cervical vagotomy or sham-unilateral cervical vagotomy. Measurements were made in the parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVHp and PVHm), SON, CeA, DMH, Arc, medial and lateral parabrachial nuclei (respectively PBm and PBl), KF, rostral, medial and caudal parts of the nucleus of the solitary tract (respectively NTSr, NTSm, NTSc). * Significantly (P<0.05) different from the contralateral side in the same operation and the same nutrition condition. AI, ad libitum, ipsilateral; AC, ad libitum, contralateral; SI, sham-operated, ipsilateral; SC, sham-operated, contralateral; VI, left cervical vagotomy, ipsilateral; VC, left cervical vagotomy, contralateral.
Fig. 4. Blackfield photomicrographs depicting c-fos mRNA hybridization signal after one hour of refeeding in rats with unilateral cervical vagotomy in the ipsilateral (ipsi) and contralateral (contr) parts of the brain at the level of the SON (A), parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVHP and PVHm, E), CeA (ipsilateral hemisphere, B, and contralateral hemisphere, F), KF, (C, ipsilateral and G, contralateral sides), the medial and lateral parabrachial nuclei (PBm and PBl, C, ipsilateral and G, contralateral sides), and in the medial (D) and caudal (H) parts of the nucleus of the solitary tract (respectively NTSm and NTSc). SCh, suprachiasmatic nucleus; ox, optic chiasm; 3v, third ventricle; 4v, fourth ventricle; AP, area postrema. Scale bars, 200 μm.
SON (Miselis et al., 1979; Kovacs & Sawchenko, 1993). Transsections effectively prevented postprandial Fos induction in the SON and significantly decreased the levels of c-fos mRNA in the magnocellular PVH ipsilateral to the transection (Figs 5 and 6). Postprandial Fos expression was also decreased in the ipsilateral Arc (Figs 5 and 6). Forebrain transections did not affect the expression of c-fos in the magnocellular PVH, SON and Arc contralateral to the lesion. In fact, c-fos mRNA expression in the regions contralateral to the lesion was

Fig. 5. Optical densities (OD) of the hybridization signal of c-fos mRNA in rats with the unilateral cut between the lamina terminalis and PVH. (A) Postprandial Fos immunoreactivity (arrows) in the ventral region of the lamina terminalis. Forebrain cut, separating lamina terminalis and hypophysiotropic hypothalamus, is shown schematically (B) and on the photomicrograph representing the horizontal brain section revealed with thionin (C). OD measurements were taken separately in the ipsilateral and contralateral SON, parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVHp and PVHm), Arc, CeA, lateral parabrachial nucleus (PBl), KF, rostral, medial and caudal parts of the nucleus of the solitary tract (NTSr, NTSm, NTSc). ★ Significantly (P < 0.05) different compared to the homologous structure on the contralateral side of the brain. MPO, medial preoptic nucleus. Scale bars, 300 μm (A) and 1 mm (C).
Fig. 6. Blackfield photomicrographs demonstrating c-fos mRNA expression in response to refeeding in rat with unilateral cut between the lamina terminalis and the neuroendocrine hypothalamus in the SON (A), parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVHp and PVHm, E), CeA, ipsilateral to cut (ipsi) hemisphere, B; contralateral to cut (contr) hemisphere, F); KF, (C, ipsilateral and G, contralateral sides), medial and lateral parabrachial nuclei (PBm and PBl, C, ipsilateral and G, contralateral sides); in the medial part of the nucleus of the solitary tract (NTSm, D) and in the Arc (H). SCh, suprachiasmatic nucleus; ox, optic chiasm; 3v, third ventricle; 4v, fourth ventricle. Scale bars, 200 μm.
Fig. 7. Direct projections from the medial part of the nucleus of the solitary tract (A–C) and from the ventral region of the lamina terminalis (D–F) to the hypothalamic areas expressing Fos during refeeding. (A) Injection site of the anterograde tracer BDA centred in the medial part of the nucleus of the solitary tract (NTSm). (B) Photomicrographs depicting fibres positive to BDA (black arrows) in proximity with Fos-immunoreactive nuclei (white arrowheads) in the medial and lateral parabrachial nuclei (PBm and PBl), CeA, and in the parvocellular part of the paraventricular hypothalamic nucleus (PVHp). (D) Injection site of BDA involving the OVLT. (E) Photomicrographs demonstrating BDA fibres (black arrows) originating from the ventral region of the lamina terminalis to the areas expressing Fos (white arrowheads) during refeeding. The nuclei illustrated are the parvocellular and magnocellular parts of the PVH (PVHp and PVHm), SON and Arc. The distribution of postprandial Fos (yellow dots), and the BDA fibres (black drawing lines) in the hypothalamus at the level of the PVH and SON is schematically represented on C, for the injections of BDA in the medial NTS, and on F for the injections of BDA in the ventral region of the lamina terminalis. ox, optic chiasm. Scale bars, 200 μm (A and D) and 20 μm (B and E).
not distinguishable from that seen in the intact refeed animals \((P > 0.05)\). The unilateral cut of the lamina terminalis fibres did not alter postprandial Fos expression in the OVLT, CeA, DMH, PVT, PBI, PBm, and the NTS (Figs 5 and 6). In control rats with unilateral transections positioned just rostral to the OVLT, postprandial expression of c-fos mRNA on the ipsilateral side of the brain did not differ from that observed on the contralateral side \((P > 0.05)\) and was similar to the expression seen in refeed intact rats.

The anterograde tracer BDA was injected into the medial part of the NTS \((n = 7)\) to reveal the structures both receiving direct projections from the NTS and expressing Fos during refeeding. In four animals, the injection sites were restricted to the NTS with a larger injection site \((n = 1)\), which reached the medial NTS and which partially diffused to the rostral NTS (a case demonstrated on Fig. 7A and B), and with smaller injection sites \((n = 3)\), which included the central, medial, and intermediate subnuclei of the medial NTS. Three rats, in which BDA injections were located dorsally to the NTS within the cuneate and external cuneate nuclei, were excluded from analyses. In four rats with successful BDA injections into the NTS, the BDA-positive fibres and buttons were found in the proximity of the Fos-ir cells seen in refeed animals, that is, in the PB nuclei, dorsomedial thalamus, and in the lateral hypothalamic area, DMH and the parvocellular PVH (Fig. 7C). Within the parvocellular PVH, the BDA fibres were running mostly through the mediodorsal subdivision. BDA fibres were not found in the magnocellular PVH, SON and Arc. In rat with a large injection site, the BDA fibres were distributed equally in both the medial and lateral PB nuclei (Fig. 7B), whereas in rats with smaller BDA injection sites, the BDA fibres were mostly observed in the lateral PB nucleus. In the medulla and pons, the ascending fibres were preferentially located ipsilateral to the injection side. Thus, ipsilateral PB demonstrated much higher density of BDA fibres, than contralateral PB. However, some fibres crossed the brainstem midline and ascended contralaterally.

In eight rats, BDA was injected in the ventral part of the lamina terminalis to delineate the potential targets of the neurons projecting from the OVLT. In three animals, the injection site included the OVLT and partially diffused into the adjacent anteroventral medieal preoptic area (Fig. 7D and E), or included the OVLT and the adjacent parts of the horizontal diagonal band and the anteroventral medieal preoptic area. In these three animals, the BDA-positive fibres and buttons were found in the parvocellular PVH, more densely in its ventral subdivision. Magnocellular PVH and SON demonstrated lower contents of BDA fibres than those seen in the parvocellular PVH, but still, on each 30-μm-thick coronal brain section sliced across these structures, branching fibres and buttons were identified (Fig. 7E). BDA-positive fibres were also found in the Arc (Fig. 7E), periventricular and dorsomedial hypothalamic nuclei, in the PVT and in the lateral hypothalamic area. No BDA-positive fibres were seen in the CeA, PB nuclei and NTS. In two cases with the injections limited mostly to one side of the ventral lamina terminalis (Fig. 7D), we saw fibres running predominantly within the hemisphere ipsilateral to injection, although some fibres crossed the ventral hypothalamus at the level of the retrochiasmatic area and travelled within the contralateral hypothalamus. Schematic representation of Fos-immunopositive cells in refeed rats and the fibres running from the ventral region of the lamina terminalis is shown on Fig. 7F.

**Discussion**

The present study provides evidence for the contribution of at least two pathways in activation of the neuroendocrine hypothalamus during refeeding. Unilateral disruption of vagal inputs decreased refeeding-induced Fos expression in the caudal and medial NTS, lateral PB, Kölliker-Fuse nucleus, central amygdala, and parvocellular part of the PVH. In these regions, direct projections from the medial NTS were detected. The NTS is a primary relay structure for the visceral signals transmitted by the vagal afferents. Receptive fields of the vagal afferents have precise viscerotopic representation in the NTS; the rostral part of the NTS is activated by the pharyngolaryngeal vagal afferents as well as the gustatory branches of the cranial nerves VII and IX, whereas the medial and caudal parts of the NTS receive the vagal afferents from the lower parts of the alimentary tract (Altschuler et al., 1989; Fraser et al., 1995). This partition of the receptive fields within the NTS is consonant with the significant decrease in the postprandial Fos expression after cervical vagotomy, which was seen solely within the ipsilateral medial and caudal NTS. UCV was performed just proximal to the recurrent laryngeal branch, leaving the superior laryngeal and pharyngeal branches intact and therefore preserving the oropharyngeal sensory signals to the rostral NTS. However, the transection spared the afferents from the oesophagus and lower gastrointestinal tract. Significant reduction in postprandial Fos expression was observed in the PBI and KF, but not in the PBm, probably because signals to the latter are mainly conveyed from the rostral NTS. The PBI receives massive stomach and gut input through the caudal NTS, but at the level of the medial NTS and its parabrachial projections, anatomical and physiological representations of the gustatory and gastrointestinal receptive fields are partially overlapped (Altschuler et al., 1989; Herbert et al., 1990; Karimnamazi et al., 2002).

UCV did not prevent postprandial Fos expression in the magnocellular PVH and SON. Direct projections from the medial NTS to the magnocellular regions were not detected in the present study, which is in agreement with reports that have shown only sparse projections from the caudal NTS to the magnocellular PVH (Cunningham & Sawchenko, 1987; Cunningham & Sawchenko, 1988). Vagal afferents are apparently involved in the transmission to the magnocellular PVH of the signals from the portal venous osmoreceptors, which monitor the alterations in local osmolality (Morita et al., 1994; Morita et al., 1997; Carlson & Osborn, 1998; Starbuck et al., 2002). However, in the case of the systemic osmotic challenge, bilateral vagotomy reduces Fos immunoreactivity in the NTS and parvocellular PVH, but failed to decrease Fos expression in the SON and magnocellular PVH (Starbuck et al., 2002). The implication of the circumventricular organs such as the SFO in the monitoring of the elevated plasma osmolality after hyperosmotic challenge or eating has been suggested (Starbuck & Fitts, 2002).

Cell groups in the OVLT and SFO, which are the forebrain circumventricular organs, are the important structures to capture and convey circulating information to the neuroendocrine hypothalamus (Kovacs & Sawchenko, 1993; Starbuck & Fitts, 2002; Honda, 2003). In the SON and magnocellular PVH, OVLT-originated fibres and terminals were detected in the vicinity of neurons activated during refeeding. The OVLT fibres were also running through the Arc, which demonstrated a decrease in postprandial Fos expression after the lamina terminalis cut. Postprandial Fos expression in the regions sharing direct projections from the medial NTS and OVLT were not affected by either UCV or ULT. These included the DMH, PVT and the lateral hypothalamic area where the postprandial Fos expression on the lesioned side was indistinguishable from that on the intact side. It is noteworthy that in the parvocellular part of the PVH, postprandial Fos expression was decreased after UCV, but not after ULT, even though, in this region, the fibres and terminals originating from the OVLT were much denser than those coming from the medial NTS.
Different transduction mechanisms based on different mediators specific for each individual input to the paraventricular PVH may be responsible for this phenomenon. Indeed, the large portion of the NTS input to the PVH is noradrenergic (Sawchenko & Swanson, 1982), whereas the neurotransmission between the OVLT and the hypothalamic neuroendocrine neurons is mediated by γ-aminobutyric acid (GABA) and glutamate (Yang et al., 1994). Norepinephrine strongly activates PVH neurons presumably via α1 adrenergic receptors (Plotzky et al., 1989; Cole & Sawchenko, 2002). In contrast, glutamate administrations into the PVH provoked only a weak increase in Fos expression (Cole & Sawchenko, 2002). GABAergic effects on the neuroendocrine neurons include both inhibitory and disinhibitory components, mediated, respectively, through the GABA-A and GABA-B receptors (Yang et al., 1994; Kolaj et al., 2000). Therefore, ascending projections from the NTS presumably activates the paraventricular PVH, whereas the effects of the lamina terminalis cell groups on the neurosecretory neurons may be inhibitory as well as excitatory. Salt loading, by, respectively, decreasing and increasing CRF expression in the paraventricular and magnocellular divisions of the PVH (Imaki et al., 1992; Kovacs & Sawchenko, 1993), represent a challenge illustrating the complex role of the OVLT in controlling gene expression within PVH neurons.

Experiments with transections of the fibres of the lamina terminalis highlighted the importance of the cell groups of the lamina terminalis in the activation of the magnocellular PVH and SON during refeeding. However, this approach does not exclude the possibility for an indirect involvement of the vagus nerve in this activation. Indeed, the hindbrain vagal complex innervates the SFO (Zardetto-Smith & Gray, 1987; Gu & Ju, 1995) and the integrity of lamina terminalis does not prevent the decrease in water intake after hyperosmotic challenge in rats with bilateral vagotomy (Starbuck et al., 2002). However, the induction of Fos expression in the SFO in response to the hyperosmotic load was not affected by bilateral vagotomy (Starbuck et al., 2002). Therefore, if interactions between the vagus and lamina terminalis exist, the mechanisms underlying such interactions remain to be elucidated.

Fos expression in the magnocellular neurons in animals refed for one hour was confined mostly to the AVP cells. The increase in plasma osmolality owing to the rise in plasma sodium and glucose during feeding (Bloom et al., 1975; Houpt et al., 1983; Gill et al., 1985) may be responsible for the activation of the AVP neurons. During feeding, physiological effects of AVP contribute to the water balance regulation, blood pressure modifications and food termination (Pittman et al., 1982; Palkovits, 1984; Langhans et al., 1991). In contrast to AVP, oxytocin neurons demonstrated in refed animals only modest levels of Fos immunoreactivity. Previously, it has been shown that Fos expression could occur in both, AVP and oxytocin neurons after ingestion of hypertonic sucrose (Naimi et al., 1997), which suggests that the activation of oxytocin neurons could depend on the nature of food. However, the consumption of sweetened condensed milk induced the expression of AVP heteronuclear (hn) RNA, but not oxytocin hn RNA in the magnocellular neurons (Naimi et al., 1997).

Refeeding induced the expression of Fos protein in approximately 30% of the CRF parvocellular neurons, mainly located in the dorsal and ventral subdivisions of the paraventricular PVH, which largely project to the preganglionic regions of the brainstem and spinal cord (Swanson et al., 1980). This result suggests that activated CRF neurons were involved rather in autonomic regulations than in the HPA axis activation. The lack of sustained activation of hypophysiotropic CRF neurons in refed animals is in agreement with a drop in the corticosterone levels during refeeding (Timofeeva et al., 2002).

In summary, the present study emphasizes the implication of the vagal afferents and lamina terminalis in the brain activations occurring following a meal. The NTS, PB, CeA and paraventricular PVH were predominantly affected by the vagus-associated postprandial signals, whereas the activation of the magnocellular PVH and SON strongly depended on the lamina terminalis output. The results of the present study provide sound evidence that the postprandial central activation requires the integrity of both the vagal- and lamina terminalis-associated pathways.

**Abbreviations**

ABC, avidin-biotin-peroxidase; Arc, arcuate hypothalamic nucleus; AVP, arginine-vasopressin; BDA, biotinylated dextran; CeA, central amygdala; CRF, corticotrophin-releasing factor; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DMH, dorsomedial hypothalamic nucleus; KF, Kölliker-Fuse nucleus; KPBS, potassium phosphate-buffered saline; NTS, nucleus of the solitary tract; OD, optical density; OVLT, organum vasculosum of the lamina terminalis; PB, parabrachial nucleus; PVH, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; SFO, subfornical organ; SON, supraoptic nucleus; UCV, unilateral cervical vagotomy; ULT, unilateral lamina terminalis cut.

**References**


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