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Research report

Effect of LPS administration on the expression of POMC, NPY, galanin, CART and MCH mRNAs in the rat hypothalamus

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Abstract

Anorexia and weight loss are manifestations of inflammation seen both in patients and in experimental animal models such as the lipopolysaccharide (LPS)-treated rat. Using in situ hybridization, the levels of mRNAs encoding proopiomelanocortin (POMC), neuropeptide Y (NPY), galanin, melanin-concentrating hormone (MCH) and cocaine- and amphetamine-regulated transcript (CART) were investigated in the rat hypothalamus after a single intraperitoneal dose (125 μg/kg) of LPS. Four hours after LPS injection the food intake was significantly decreased. POMC and CART mRNA levels were increased in the arcuate nucleus, and MCH, CART and galanin mRNAs were all decreased in the lateral hypothalamic area in LPS-treated rats. Levels of mRNAs for NPY and galanin in the arcuate nucleus, and for MCH and CART in the zona incerta did not change significantly after LPS treatment. These findings support the hypothesis that LPS-induced factors mediate signalling to the POMC/CART neurons in the arcuate nucleus which could lead to reduced food intake by decreasing MCH, CART and galanin synthesis in target lateral hypothalamic neurons.

Theme: Endocrine and autonomic regulation

Topic: Neural–immune interactions

Keywords: Anorexia; Arcuate nucleus; Feeding; Inflammation; Lateral hypothalamus; Leptin; Neuropeptides

1. Introduction

A marked decrease in food consumption is a consequence of infection and inflammatory processes (see [49]). Peripheral administration of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a commonly used experimental model to mimic inflammation, and induces an anorectic response in rats [47]. This response may be produced by release of a number of interacting cytokines such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) from activated immune cells, mainly macrophages and monocytes (see [3,49]). Potential mechanisms by which LPS and peripheral cytokines can influence the CNS have been described pointing out the hypothalamus as a likely site of action (see [16,49]).

Recent research has unravelled an intricate hypothalamic circuitry of neuropeptide-defined neuronal populations regulating feeding behaviour. A key component of this circuitry is the arcuate nucleus which contains two cell populations that influence food intake antagonistically. One group of neurons express pro-opiomelanocortin (POMC), which is cleaved into the anorexigenic α-melanocyte-stimulating hormone (α-MSH) [19,51]. A second group of cells expresses neuropeptide Y (NPY) which is orexigenic [10,61]. Both these neuronal populations express receptors for the satiety-inducing adipocyte-derived hormone leptin [8,43] and project in parallel into the brain [7]. One of the targets for the arcuate nucleus projections are neurons in the lateral hypothalamic area (LHA) which express melanin-concentrating hormone (MCH) [7,15], another feeding-stimulatory peptide [52,55]. Both the POMC- and the MCH-containing cells

Abbreviations: α-MSH, α-Melanocyte-stimulating hormone; CART, Cocaine- and amphetamine-regulated transcript; IL, Interleukin; LHA, Lateral hypothalamic area; LPS, Lipopolysaccharide; MCH, Melanin-concentrating hormone; NPY, Neuropeptide Y; POMC, Pro-opiomelanocortin; TNF-α, Tumour necrosis factor-α

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also coexpress cocaine- and amphetamine-regulated transcript (CART; [5,14,65]), which is transcribed into peptides that induce satiety when injected intracerebroventricularly (i.c.v.) [6,33,36]. Finally, both the LHA and the arcuate nucleus contain neurons expressing galanin, a peptide which has also been demonstrated to promote food intake with special bias towards fat ingestion [34].

In this study, we used in situ hybridization to further explore if these neuropeptide systems are involved in the anorexia response to inflammation. Thus, the effect of a single dose of LPS on mRNAs encoding NPY, POMC, MCH, galanin and CART was studied in the rat hypothalamus using in situ hybridization.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (250–300 g; B&K Universal) were used. The animals were kept under regular lighting conditions (light cycle 6.00–18.00) in a temperature-controlled environment with free access to standard rodent chow and tap water. The experiments had been approved by the local ethical committee, Stockholms norra djurförsöksetiska nämnd. Prior to the experimental day, rats were individually housed in acrylic cages for study of food intake by weighing food pellets before and 4 h after LPS administration. Rats were injected intraperitoneally with LPS from *Salmonella abortus equi* (Sigma, St Louis, MO; 125 μg/kg; $n=4$) or vehicle (0.9% NaCl; $n=4$) at 18.00 h, at the onset of the dark phase when rats express spontaneous feeding [31]. Four hours after injection, the rats were killed by decapitation and their brains rapidly frozen on dry ice. Coronal sections (14-μm-thick) were cut in a cryostat (Microm), thawed onto 'Probe On' slides (Fisher Scientific) and stored at −20°C until hybridization.

2.2. In situ hybridization

Oligonucleotide probes were synthesised by Scandinavian Gene Synthesis AB. The oligonucleotide sequences were complementary to the nucleotides 1671–1714 of NPY mRNA [37], 266–319 of POMC mRNA [13], 223–270 of CART mRNA [12], 479–527 of MCH mRNA [46], and 70–189 of galanin mRNA [66]. The probes were labelled at the 3′-end as previously described [11] using terminal deoxynucleotidyl transferase (Amersham) in a cobalt-containing buffer with [^{35}S]dATP (New England Nuclear) and purified with Quiaquick Nucleotide Removal Kit (QIAGEN). Tissue sections were air-dried and incubated for 16–18 h at 42°C in humidified boxes with 10^6 c.p.m. labelled probe per 100 ml of hybridization cocktail containing 50% deionized formamide (J. T. Baker Chemicals BW), 4×standard saline citrate (SSC; 1×SSC=0.15 M NaCl and 0.01 M sodium citrate), 1×Denhardt's solution (0.02% each of bovine serum albumin, Ficoll (Pharmacia) and polyvinyl pyrrolidone), 0.2 M $NaPO_4$ (pH 7.0), 1% N-lauroylsarcosine, 10% dextran sulphate (Pharmacia), 500 mg/ml denaturated Salmon testis DNA (Sigma) and 200 mM dithiothreitol (LKB). An excess (100×) of the appropriate cold probe was added to the incubation cocktail of control sections to determine specificity of labelling. After hybridization, the sections were rinsed in 1×SSC, four times for 15 min at 55°C, followed by 30 min at room temperature, immersed in distilled water and air-dried. The slides were dipped in NTB2 nuclear track emulsion (Kodak) diluted 1:1 with distilled water, exposed in the dark at −20°C for 1 to 28 days depending on the probe, developed in D19 (Kodak) and analysed in a Nikon Microphot-FX microscope. Photomicrographs were taken with Kodak T-max 100 film.

2.3. Quantification

The number of grains overlying labelled cells was counted on Cresyl Violet-stained sections observed in a Nikon Microphot microscope. The arcuate nucleus, LHA, perifornical hypothalamic area (PHF) and zona incerta, from level bregma −2.5 mm to bregma −1.9 mm were examined. Every sixth section of a series was selected (in total 10 sections of each hypothalamus from four animals in each group). Cells were considered labelled when the number of silver grains overlying the cytoplasm exceeded five times the background levels. All labelled cells were included for counting. Thus, for each neurochemically defined cell population, a total of at least 300 neurons labelled with each of the neuropeptide mRNAs markers was counted. The results were evaluated statistically using the paired Student's *t*-test (Statistica, StatSoft Inc.).

3. Results

In rats injected with LPS, behavioural changes were observed as compared to saline-treated controls. Mean food intake 4 h after injection was significantly decreased (−68%; $P<0.01$). Similarly, but not quantified, we observed a decrease in drinking, as well as general locomotor activity. Piloerection could also be observed, and LPS-treated animals appeared uninterested in their surroundings.

Four hours after LPS injection, several changes in hypothalamic gene expression could be detected by in situ hybridization. Fig. 1 illustrates the anatomical regions investigated in this experiment. In arcuate nucleus cells, POMC mRNA levels were increased to 164.5±6.2% of control ($P<0.01$), while NPY (105.1±12.4%) and galanin (88.6±9.2%) gene expression showed no significant difference. However, in the ventral part of the LHA, galanin mRNA signal was decreased in neuronal profiles (71.2±8.8% of control, $P<0.05$). Cellular expression of

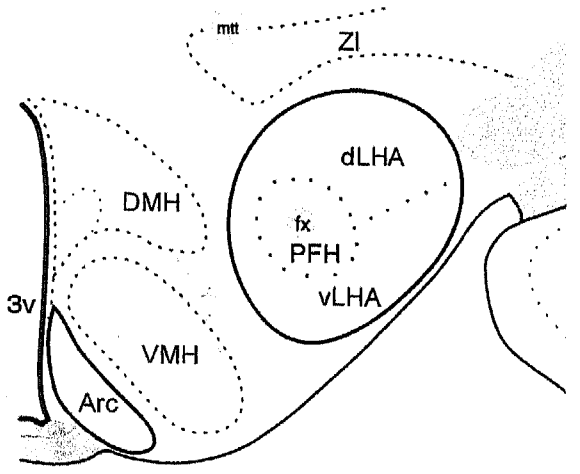

Fig. 1. Schematic drawing adapted from Swanson [63] delineating anatomical regions investigated in this study. POMC- and CART-expressing cells are found in the arcuate nucleus (Arc). CART-containing cells, as well as cells expressing MCH, are also found in the dorsal part of lateral hypothalamic area (dLHA), perifornical area (PFH) and zona incerta (ZI); however, LPS-induced decreases in these mRNAs were only seen in the dLHA. Galanin mRNA-containing neurons were seen in both the Arc and the ventral part of lateral hypothalamic area (vLHA); in the latter region LPS treatment decreases galanin expression. 3v, third ventricle; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; fx, fornix; mtt, mammillothalamic tract.

CART was increased in the arcuate nucleus (148.3±6.2% of control, $P<0.01$), but decreased in the dorsal part of the LHA (75.1±9.8% of control, $P<0.05$), whereas no significant change could be detected in the zona incerta (93.2±8.7%). MCH mRNA levels were decreased in LHA cells (64,5±7.5% of control, $P<0.05$), but showed no significant difference in the zona incerta after LPS treatment (88.2±9.3%). For illustration, see Figs. 2–4. Notably, although LPS administration affected signal intensity for several mRNAs (as described above), there was no significant change in the mean number of labelled neurons per section for POMC, NPY, galanin, CART or MCH in any of the regions studied (data not shown). Hybridization in the presence of an excess of cold probe abolished labelling patterns, indicating specificity.

4. Discussion

In the present study we demonstrate that an i.p. LPS injection sufficient to elicit hypophagia (present results) also causes increases in mRNA levels for the anorexigenic messengers POMC [19,51] and CART [6,33,36] in the arcuate nucleus, and decreases in mRNAs encoding the feeding-stimulatory peptides MCH [52,55] and galanin [34] in the LHA. The following discussion focuses on some potential mechanisms underlying these changes.

There is increasing evidence that the melanocortinergic projection from the arcuate nucleus is of particular importance in inhibiting food intake. Both the endogenous α-MSH [51] and melanocortin agonists [19] induce satiety when administered i.c.v. Furthermore, obesity has been reported both in animals rendered genetically deficient of ligands [69] and receptors [30] of the melanocortin system. In the LPS-treated animal, α-MSH has been suggested to both mediate the anorexia and antagonise the febrile response typical of this model [28,29,41,45]. A prominent regulator of POMC gene expression is the adipocyte-derived hormone leptin [44,59,64,70]. Administration of LPS increases expression and circulating levels of leptin

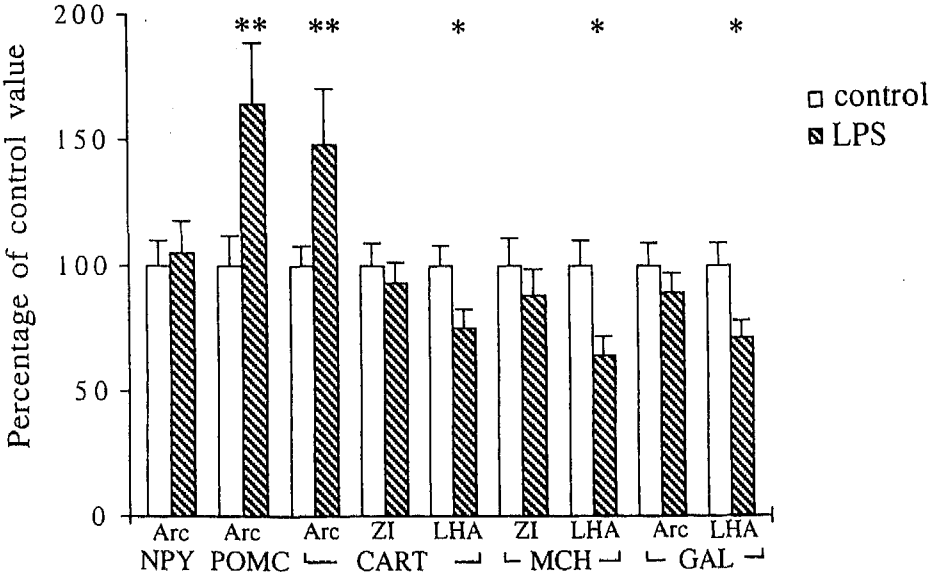

Fig. 2. Effects of saline (control; open bars) and LPS-treatment (striped bars) on NPY, POMC, CART, MCH and galanin (GAL) mRNA levels (number of grains/cell; see text). The data are presented as mean±S.E.M. *$P<0.05$; **$P<0.01$ compared to control. Arc, arcuate nucleus; LHA, lateral hypothalamic area; ZI, zona incerta.

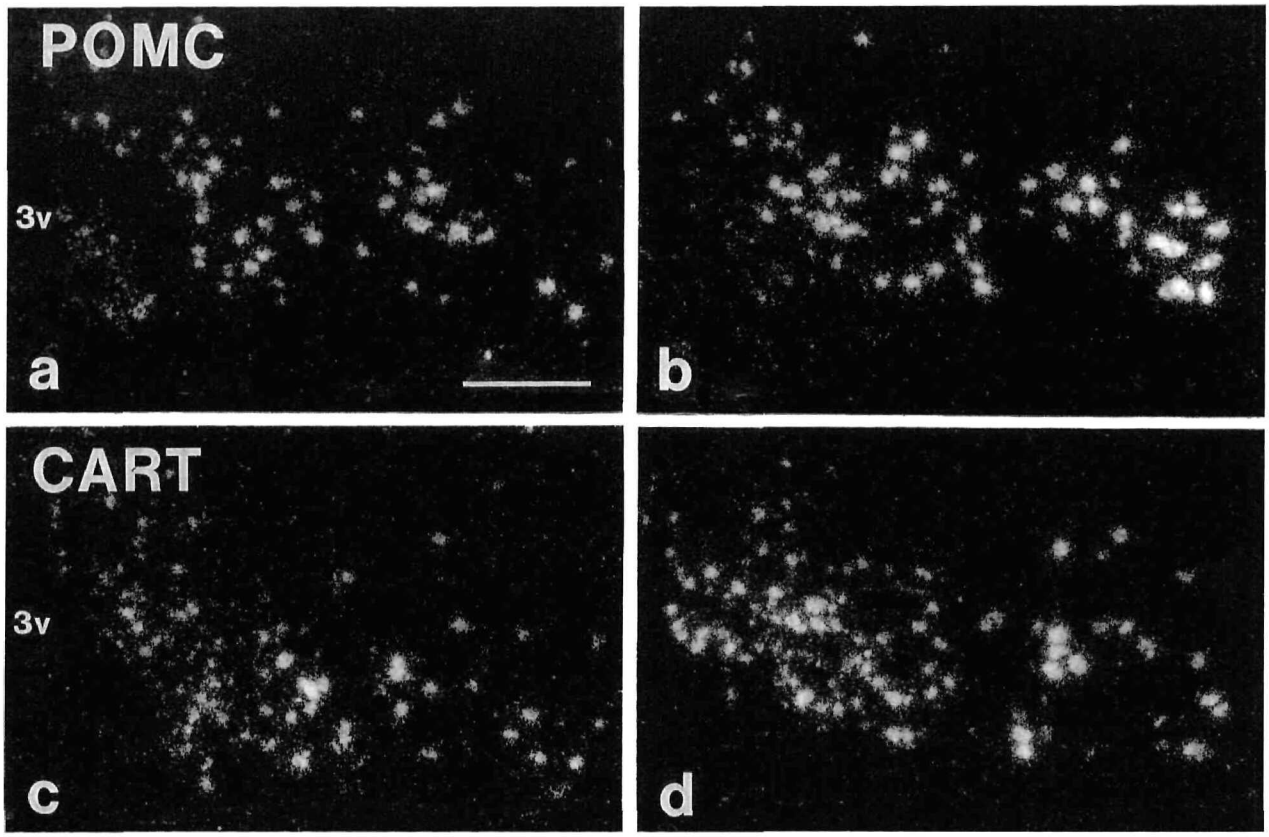

Fig. 3. Dark-field micrographs of sections from the arcuate nucleus following hybridization for POMC (a,b) and CART (c,d) in saline-(a,c) and LPS-(b,d) treated rats. There is increase in POMC and CART mRNA levels 4 h after LPS administration. 3v, third ventricle. Scale bar=200 μm.

[21,23,57], likely through an intermediate step involving induction of proinflammatory cytokines such as TNF-α [20] and IL-1 [18]. Thus, a plausible chain of events would be that an endotoxin-induced increase in circulating cytokines causes an elevation of plasma leptin, which in turn stimulates POMC/CART expression in the arcuate nucleus.

However, the hypothalamic mRNA changes seen after LPS injection (present data) are in some aspects different from those observed following leptin injection. Here, LPS administration had no effect on the level of NPY mRNA in the arcuate nucleus, whereas one of the most potent effects of leptin, when administered to the leptin-deficient *ob/ob* mouse, is to inhibit NPY gene expression [58,62]. Regarding MCH, the data are less conclusive. Leptin has been reported to decrease MCH expression [56], and MCH mRNA levels in *ob/ob* mice are higher than in controls [52]. These data would agree well with the LPS effect reported here as mediated by leptin. However, in contrast, another study reports leptin-induced increases in the hypothalamic content of MCH mRNA and peptide [27]. These discrepancies suggest that LPS may influence gene expression, at least partly, through mediators other than leptin, in agreement with the demonstration that LPS-induced anorexia can also be observed in *ob/ob* mice [18]. Candidate mechanisms for this include direct effects on the hypothalamus of IL-1 [17,54,67], IL-6 [60,68], TNF-α, or even of LPS itself [4,35], since specific receptors for all these messengers are present in the brain and may be up-regulated following endotoxin shock (see [49]). (However, direct effects of LPS receptor activation on hypothalamic peptide transcription appear less likely in view of the demonstration that LPS given i.c.v. does not affect POMC mRNA levels [22,50]).

Microbial infection also activates the hypothalamo–pituitary–adrenal axis, increasing serum glucocorticoid levels [25,53]. Both POMC- and NPY-expressing neurons in the arcuate nucleus contain glucocorticoid receptors [9,26] and increase the expression of their respective peptides when these receptors are stimulated [2,38,42]. Thus, the POMC mRNA increase which we observed after LPS administration could also partly be the result of a stress response, and a similar mechanism could be counteracting the expected effect of LPS on NPY expression (see above). Finally, an alternative explanation for our negative finding with NPY is that the time interval chosen in the present study may have been too short for a transcriptional decrease to be detectable.

MCH neurons in the LHA receive innervation from the arcuate nucleus POMC population [15], and may thus be under transcriptional melanocortinergic control. Notably, increases in MCH gene expression have been reported in

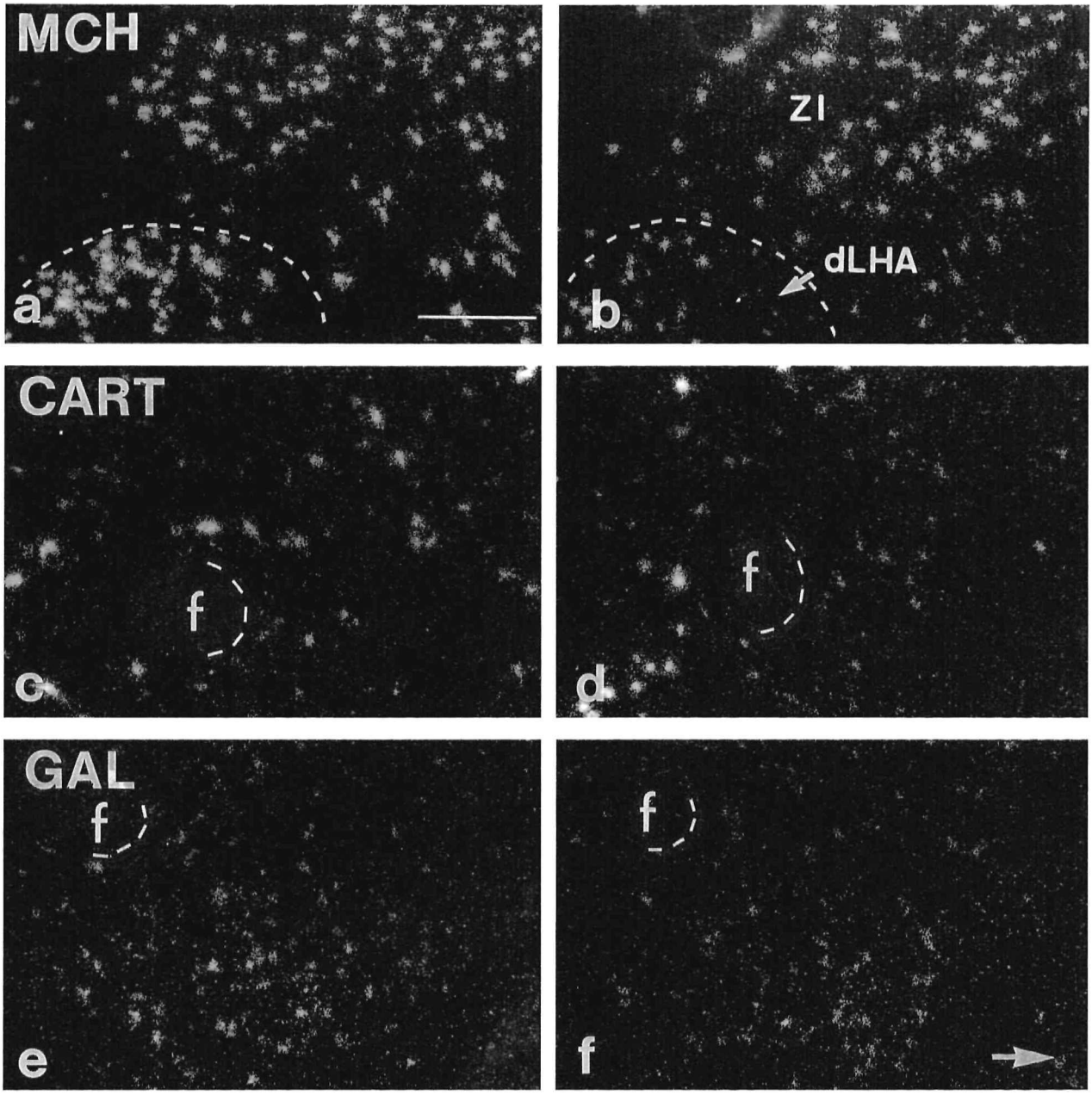

Fig. 4. Dark-field micrographs of sections from the LHA following hybridization for MCH (a,b), CART (c,d) and galanin (GAL; e,f) in rats treated with saline (a,c,e) and LPS (b,d,f). Following LPS administration, MCH mRNA labelling intensity is decreased in neurons located in the dorsal part of the lateral hypothalamic area (dLHA; delineated by stippled line), whereas MCH expression in the zona incerta (ZI) is not affected. Note also LPS-induced decreases in the levels of CART and galanin in the dorsal and ventral part of the LHA, respectively. Arrow in (f) points in lateral direction. f, fornix. Scale bar=200 μm.

rodents after both genetic and pharmacological melanocortin blockade [24], and Ludwig et al. [40] have demonstrated an antagonistic relationship between melanocortins and MCH on feeding. Thus, decreased MCH expression in the LPS-challenged rat may be due to an increased inhibitory melanocortinergic tone.

Little is known about the function of the galanin neurons in the PFH which were affected by LPS treatment (present data). Their localisation within a brain region long established as a 'feeding center' [1] would agree well with a role in the control of food intake. However, available data would rather suggest that the orexigenic effects of galanin are exerted by neurons in the paraventricular hypothalamic nucleus [39]. It may be speculated that the PFH galanin is involved in the pyretic response to endotoxinaemia [48], but this remains to be investigated.

CART is coexpressed with POMC in the arcuate nucleus [14,65], and with MCH in the LHA [5,65]. We were intrigued to find that the same stimulus, i.e. LPS administration, had opposite effects on CART expression in these

two populations, by increasing CART levels in the arcuate nucleus and decreasing them in the LHA. The presence of CART in multiple hypothalamic cell populations [6,12,32] has made it difficult to localize CART-induced hypophagia anatomically, as this effect has been investigated only through intracerebroventricular injections [33,36]. The present data are the first to show a differentiated regulation of CART in an anorectic context. Notably, CART regulation was seen to follow the direction of the LPS-induced modulation of the neuropeptide messenger characteristic of the two cell groups, i.e. POMC and MCH. Speculatively, it may be hypothesized that CART plays a role as an autoregulatory messenger regulating excitability presynaptically in these populations.

In conclusion, the present results suggest that endotoxinaemia is parallelled by neurochemical hypothalamic alterations which switch the metabolic set-point of the animal to an anorectic state. Furthermore, these data may shed new light on the role of CART in food intake regulation.

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