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Separate populations of neurons within the paraventricular hypothalamic nucleus of the rat project to vagal and thoracic autonomic preganglionic levels and express c-Fos protein induced by lithium chloride

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Abstract

The role of different hypothalamic nuclei, particularly the paraventricular nucleus (PVN), in the control of food intake and feeding behaviour is well known. It is also well established that lithium chloride (LiCl) causes various disorders in feeding behaviour. In this study, we analyzed the precise distribution of hypothalamic neurons activated by i.p. LiCl administration (LCA neurons) and compared it to that of hypothalamic neurons which project to autonomic preganglionic levels (HAP neurons). We also analysed the possibility that some neurons belong to both populations of nerve cells. To this end, a multiple-labelling technique, using two retrograde fluorescent tracers together with c-Fos-like immunohistochemistry, was performed. Fast Blue was injected in the dorsal motor nucleus of the vagus and Fluorogold (FG) in the thoracic intermedial-lateral cell column, to trace parasympathetic and sympathetic pathways, respectively. LiCl was used as stimulus for c-Fos-like immunohistochemistry. HAP neurons were located mainly in the dorsal, ventral and lateral regions of the parvocellular PVN, while LCA neurons were observed predominantly in the magnocellular region of the PVN rostrally to HAP neurons. A significant number of FG/Fos double-labelled neurons were located in the dorsal parvocellular subnucleus of the PVN (dp) in the LiCl-stimulated rats. We concluded that there is a clear segregation of LCA neurons from HAP neurons within the PVN. The presence of FG/Fos double-labelled neurons in the dp suggests that this nucleus could mediate a sympathetic response after LiCl administration.

Keywords: Paraventricular hypothalamic nucleus; Lithium chloride; Fos; Fast Blue; Fluorogold; Feeding behavior; Autonomic nervous system; Rat

1. Introduction

The paraventricular hypothalamic nucleus (PVN) is a major site of neurogenic and humoral control of feeding behaviour (Luiten et al., 1987; Krukoff et al., 1994). This nucleus can be subdivided into magnocellular and parvocellular subnuclei (Swanson and Kuypers, 1980). The nerve cells of these divisions are connected with different structures which are involved in various physiological responses (Swanson and Kuypers, 1980; Swanson and Sawchenko, 1980; Roberts et al., 1993). Thus, it has been reported that the axons of PVN parvocellular neurons project to areas of the central nervous system which are involved in the regulation of autonomic functions (Swanson and Sawchenko, 1983; Luiten et al., 1987; Loewy and Haxhiu, 1993), while PVN magnocellular neurons mainly produce vasopressin or oxytocin and send their axons to the neurohypophysis (Brownstein et al., 1980; Rhodes et al., 1981; Miyata et al., 1995).

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The precise cytoarchitecture of different neuronal networks within the same anatomical structure can be established by performing multiple neuronal labelling techniques in the same experimental animal (Portillo and Pásaro, 1988). Neuronal retrograde fluorescent labelling in combination with various immunohistochemistry techniques has been so used (Sawchenko and Swanson, 1982).

The analysis of c-Fos protein expression has proven useful in studying the physiological activity of nerve cells (Dragunow and Robertson, 1988; Morgan and Curran, 1991; Krukoff, 1993). It is also known that intraperitoneal (i.p.) injection of lithium chloride (LiCl) causes various disorders in feeding behaviour, such as conditioned taste aversion (Schafe et al., 1995; Swank et al., 1995), pica (McCutcheon et al., 1992), anorexia (Dess and Vanderweele, 1994; Ervin et al., 1995), or emesis (Gu et al., 1993; Ervin et al., 1995). c-Fos-stained cells have been described in PVN, supraoptic and other hypothalamic nuclei after i.p. LiCl administration in rats (Yamamoto et al., 1992; Gu et al., 1993). The excitation of central neurons following i.p. injection of LiCl has been suggested to be mediated via vagus (parasympathetic) and splanchnic (sympathetic) nerves, as well as by humoral factors (Arnedo et al., 1991; Yamamoto et al., 1992). Of importance for the present work is the observation that certain PVN neurons project to autonomic preganglionic levels related to the control of organs involved in body metabolism, such as liver, pancreas and adrenal glands (Loewy and Haxhiu, 1993; Portillo et al., 1996). Thus, PVN hypothalamospinal (HS) and hypothalamobulbar (HB) neurons have been described projecting to the intermedial-lateral cell column (ICC) in the thoracic T8–T9 spinal cord and to the dorsal motor nucleus of the vagus (DMX), respectively (Portillo et al., 1996).

The aim of this study was to compare, in the rat hypothalamus, the precise location of neurons activated by i.p. LiCl administration (LCA neurons), with that of neurons, which project to thoracic ICC and DMX (HAP neurons). Our goal was to determine whether LCA neurons are involved in these autonomic efferent pathways. We performed Fluorogold (FG) and Fast Blue (FB) neuronal retrograde fluorescent labelling, together with c-Fos-like immunohistochemistry, in the same animal.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (350–450 g; $n = 8$), purchased from the Central Service of Experimental Animals (University of Cadiz), were used. Animals were individually housed at room temperature on a 12 h light–dark schedule (lights on at 08:00 h) with food and water ad libitum.

2.2. Neuronal tracer labelling

Under general anaesthesia (ketamine 45 mg/kg and diazepam 3 mg/kg, i.p.), animals were placed and clamped in a stereotaxic frame (Kopf, Tujunga, CA). A dorsal laminectomy was performed at thoracic level, T8–T9. Subsequently, 150–200 nl of FG were injected bilaterally into the ICC (0.5 mm from the midline and 1 mm under the dorsal surface), using a micropipette attached to a 1 μl Hamilton microsyringe. One week later, the animals were re-anaesthetised and placed in the stereotaxic frame, their heads were positioned to give access to the occipital part and a partial occipital craniotomy was performed. Afterwards, 150–200 nl of FB were injected bilaterally into the dorsomedial medulla in the DMX (0.5 mm rostral of the calamus scriptorius, 0.5 mm from the midline and 1 mm under the dorsal surface), using the same procedure.

2.3. Immunohistochemistry

A total of 1 week after the surgical procedure, food and water were removed from cages at 17:00 h. The next day, rats were injected i.p. with a LiCl solution dissolved in buffered saline (3%, 3 ml/kg, i.e. 2228 μmol/kg; $n = 6$). Control rats were injected with NaCl saline solution (4.14%; $n = 2$) using the same volume as for LiCl. The two solutions were equimolar and physiologically hypertonic (Gu et al., 1993). A total of 2 h after the injections, the animals were deeply anaesthetised with a lethal dose of barbiturate and perfused via the ascending aorta with 500 ml of 0.9% phosphate-buffered saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered solution as fixative. Brains and thoracic spinal cords were removed and postfixed in the paraformaldehyde solution for 2 h at room temperature, placed in a cryo-protective 30% buffered sucrose solution overnight at 4°C and sliced into 50 μm coronal sections on a freezing microtome (Leitz). Slices containing the hypothalamus were placed in PBS in a multiwell culture plate (24-well).

c-Fos expression in brain slices was determined using an antibody and avidin–biotin–peroxidase method. Briefly, free-floating brain sections were incubated for 24 h at 4°C in a solution of sheep polyclonal IgG against pan fos oncoprotein (Genosys). The antibody was diluted 1:3000 in 0.1 M phosphate-buffer with 1.5% normal rabbit serum. Brain sections were rinsed in PBS and incubated for 1 h at room temperature with a

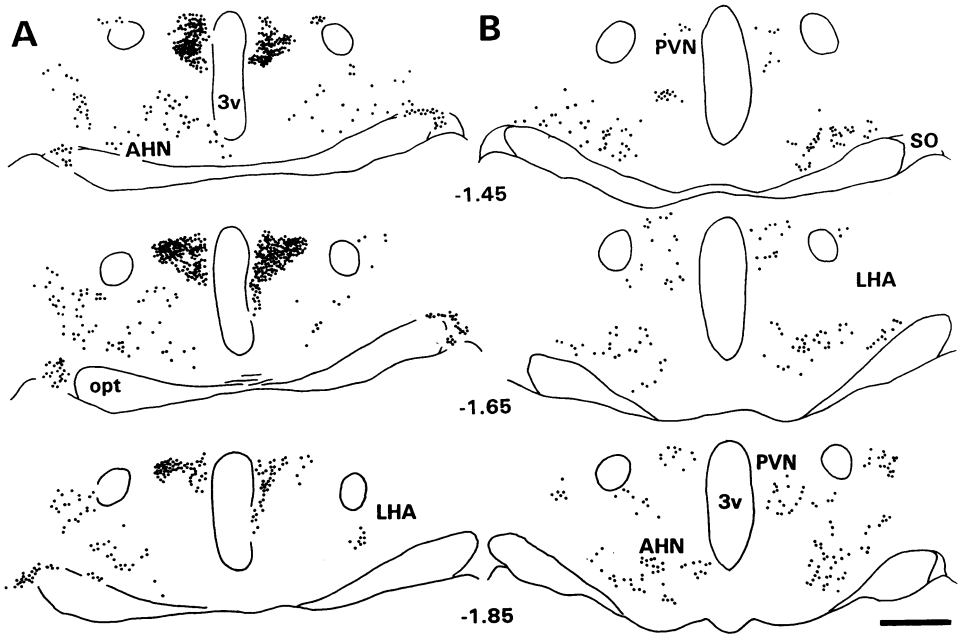

Fig. 1. Camera lucida drawing showing the distribution of c-Fos-stained neurons through the hypothalamus of a representative lithium-chloride-stimulated rat (A) and control rat injected with saline solution (B). Numbers indicate (mm) the rostrocaudal positions of sections, zero being the bregma. AHN, anterior hypothalamic area; opt, optic chiasm; SO, supraoptic nucleus; PVN, paraventricular hypothalamic nucleus; LHA, lateral hypothalamic area; 3v, third ventricle. Bar = 1 mm.

secondary biotinylated rabbit anti-sheep IgG diluted 1:200 (Vector Laboratories). Sections were incubated at room temperature for 2 h with an avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector). After rinsing in PBS, brain sections were incubated with a peroxidase substrate kit solution Vector VIP (Vector), mounted on gelatin-coated slides, dehydrated and coverslipped with DPX (Fluka). Anatomical landmarks of cerebral structures and injection sites were determined by counterstaining one of three sections according to the atlases of Paxinos and Swanson (Paxinos and Watson, 1986; Swanson, 1992).

2.4. Quantitative analyses

The number of c-Fos-stained hypothalamic neurons was determined using the transmitted light system of an Olympus BX 60 microscope with a camera lucida. Likewise, fluorescent-labelled neurons were examined using the reflected light fluorescent system in the same microscope, equipped with a filter system U-MNU (dichroic mirror DM400, exciter filter BP360–730, barrier filter BA420). FG-labelled neurons show a golden yellow fluorescent cytoplasm when excited with ultraviolet light of 360 nm wavelength (Schmued and Fallon, 1986). FB-labelled neurons show a blue fluorescent cytoplasm when excited with the same light (Kuypers et al., 1983). Correct placement of the injection sites was verified. Data are expressed as mean values of the stimulated groups.

3. Results

Analysis of hypothalamic structures showed HAP neurons throughout the rostrocaudal extension of the PVN, −1.3 to −2.3 mm from bregma and scattered cells in the caudal lateral hypothalamic area, −2.1 to −3.0 mm from bregma (references taken from Swanson's atlas, plates 24–29). A marked increase in the number of cell nuclei showing Fos-like immunoreactivity was observed in the magnocellular division of the PVN and supraoptic nuclei in LiCl-stimulated animals when compared with control rats injected with saline solution. No significant differences were detected in the rest of the hypothalamic structures analysed (Fig. 1).

The highest density of fluorescent HAP neurons was found in the caudal two-thirds of the PVN (−1.55 to −2.3 mm from bregma), mainly in the medial and lateral parvocellular subnuclei of the PVN. HAP neurons were also found in the dorsal parvocellular, posterior magnocellular subnuclei of PVN and perifornical nucleus. LCA neurons were found mostly in the rostral part of the PVN (−1.3 to −1.65 mm from bregma), in considerable number in the posterior magnocellular subnucleus of PVN, and in a lesser quantity in the dorsal and medial parvocellular subnuclei (Fig. 2 and Table 1).

Thus, HAP and LCA populations of labelled neurons overlapped within the PVN from −1.45 to −1.85 mm from the bregma, although HAP neurons were located in the most dorsal and ventral part, surround-

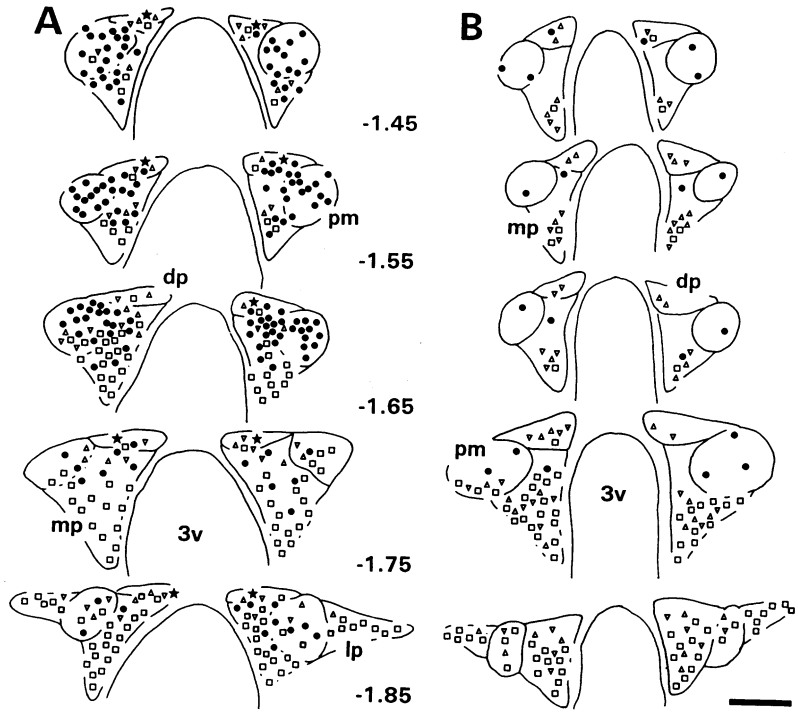

Fig. 2. Series of camera lucida drawings, showing the distribution of Fluorogold hypothalamospinal (open triangles) and Fast Blue hypothalamobulbar (open squares) retrograde-labelled nerve cells and c-Fos (black dots) immunostained neurons within the paraventricular nucleus (PVN) at the levels where these neuron populations overlapped. Stars represent FG/Fos double-labelled cells. (A) Representative lithium-chloride-stimulated animal. (B) Representative control animal injected with saline solution. Each symbol represents a maximum of ten cells. Numbers indicate (mm) the rostrocaudal position of the sections, zero being the bregma. dp, dorsal parvocellular subnucleus of the PVN; mp, medial parvocellular subnucleus of the PVN; lp, lateral parvocellular subnucleus of the PVN; pm, posterior magnocellular subnucleus of the PVN; 3v, third ventricle. Bar = 500 μm.

ing LCA neurons (Figs. 2A and 3). The dorsal population of HAP neurons within the PVN was mostly FG-labelled neurons, that is, nerve cells projecting to thoracic T8–T9 spinal cord (HS neurons). In contrast, the ventral population of HAP neurons within the PVN was mainly FB-labelled neurons, that is, nerve cells projecting to DMX (HB neurons) (Figs. 2 and 4).

3.1. Double-labelled neurons

Scattered FB/Fos and FG/Fos double-labelled cells were found in both LiCl-stimulated and control animals. In LiCl-stimulated animals, FB/Fos double-labelled neurons were found in parvocellular subnuclei (mean number = 0.6, range 0–2) and in the posterior magnocellular subnucleus of the PVN (mean number = 1, range 0–2). Some FG/Fos double-labelled neurons were also found in the anterior parvocellular (mean number = 2, range 0–4) and posterior magnocellular subnuclei of the PVN (mean number = 1, range 0–3) (Table 1). No significant differences were observed when compared to control animals (data not shown).

However, a significant number of FG/Fos double-labelled neurons were located in the dorsal parvocellular subnucleus of the stimulated rats (mean number = 9, range 1–23), while only one FG/Fos double-labelled cell was found in control animals (Fig. 3).

No double-labelled neurons were detected in the rest of the hypothalamic structures analyzed.

4. Discussion

Lithium chloride is a compound that dramatically decreases feeding behaviour in animals (Ervin et al., 1995; Swank et al., 1995). We could thus infer that the nerve centres which are activated by administration of LiCl are related to the control of feeding behaviour. The LiCl dose used in the present study (2228 μmol/kg) was chosen for its capability to induce anorexia (Ervin et al., 1995). The presence of LCA neurons within the PVN and supraoptic nucleus shown in this report is in accordance with recent studies using similar amounts of LiCl (Yamamoto et al., 1992; Gu et al., 1993).

The PVN has been described as a highly complex nucleus involved in the hypothalamic control of the autonomic nervous system (Swanson and Sawchenko, 1980; Luiten et al., 1987). Recently, our laboratory has shown that the PVN is the main efferent pathway from the hypothalamus to the autonomic preganglionic levels

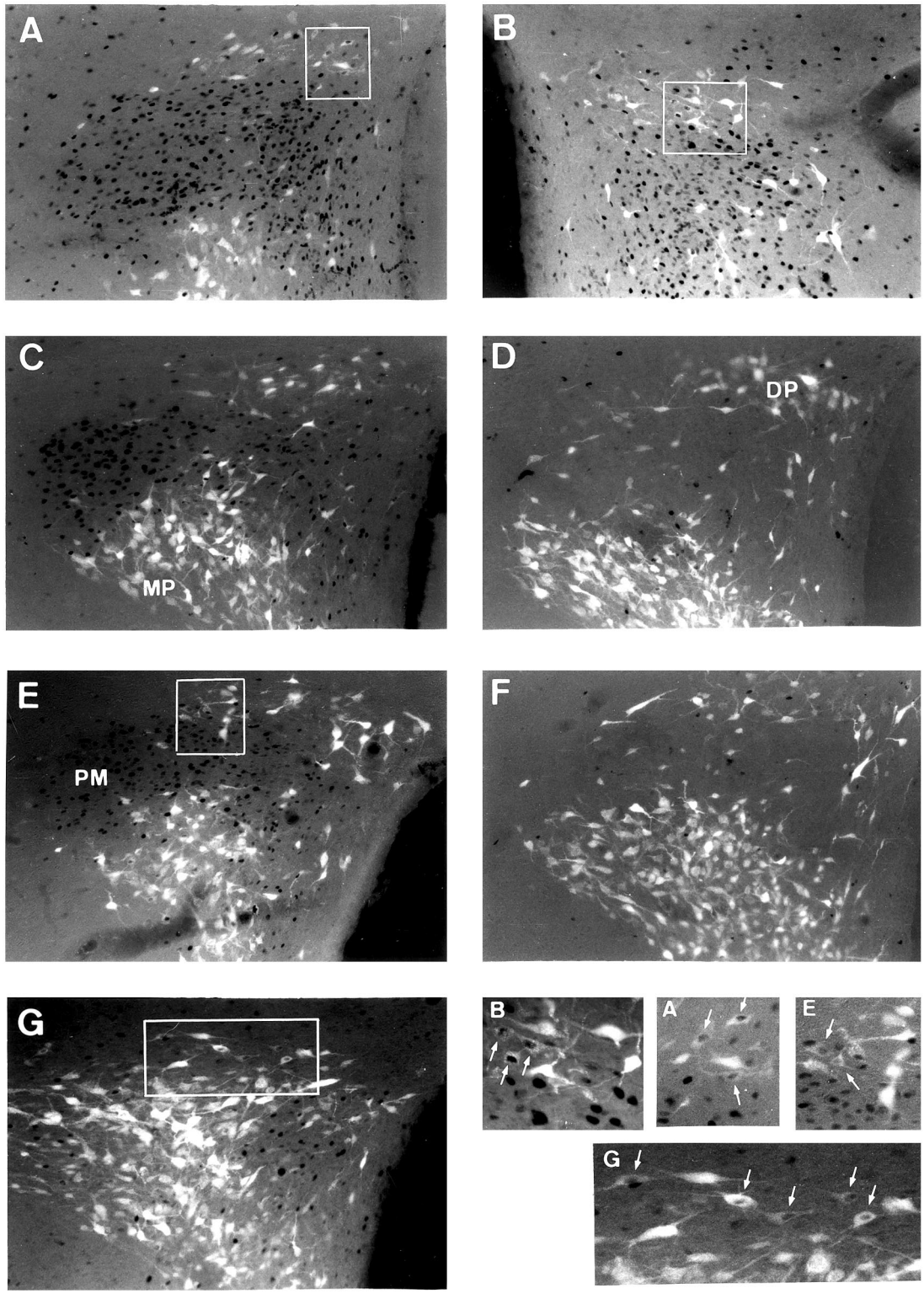

Fig. 3. Photomicrographs (200 ×) of the rat paraventricular hypothalamic nucleus, showing the distribution of c-Fos-stained and Fast Blue (FB) and Fluorogold (FG) retrograde-labelled neurons. FB was injected into the dorsal motor nucleus of the vagus, and FG into the thoracic spinal cord. Fluorescent-labelled and c-Fos-stained neurons, after lithium chloride administration, are shown in A, B, C, E and G. Neurons labelled after control saline solution administration are shown in D and F. High magnification (400X) of white frames in the dorsal parvocellular subnuclei are shown at the bottom right. White arrows indicate FG/Fos double-labelled cells. A and B − 1.55 mm from bregma. C and D − 1.6 mm from bregma. E and F − 1.65 mm from bregma. G − 1.8 mm from bregma. DP, dorsal parvocellular subnucleus of the PVN; MP, medial parvocellular subnucleus of the PVN; PM, posterior magnocellular subnucleus of the PVN.

Table 1
Summary of the number of labelled cells found in the different nuclei and paraventricular subnuclei of the rat hypothalamus[a]

	Fos	FB	FG	FB/Fos	FG/Fos
	Mean (range)	Mean (range)	Mean (range)	Mean (range)	Mean (range)
Anterior parvocellular	42 (19–83)	4 (1–7)	18 (6–30)	—	2 (0–4)
Dorsal parvocellular	86 (46–108)	43 (9–84)	178 (146–225)	0.6 (0–1)	9* (1–23)
Medial parvocellular	435 (372–519)	537 (226–1139)	226 (185–280)	1 (0–2)	—
Lateral parvocellular	5 (0–12)	48 (12–120)	21 (6–30)	0.3 (0–1)	—
Anterior magnocellular	75 (46–114)	9 (5–17)	20 (13–27)	—	—
Posterior magnocellular	811 (642–980)	88 (35–186)	110 (75–151)	1 (0–2)	1 (0–3)
Anterior hypothalmic area	240 (34–438)	—	—	—	—
Lateral hypothalmic area	285 (21–536)	—	—	—	—
Supraoptic nucleus	230 (177–279)	—	—	—	—

Fos, c-Fos-stained neurons; FB, Fast Blue labelled neurons; FG, Fluorogold-labelled neurons; FB-Fos, Fast Blue and c-Fos double-labelled neurons; FG-Fos, Fluorogold and c-Fos double-labelled neurons.
The significant number of FG-Fos double-labelled neurons in the dorsal parvocellular subnucleus of the PVN is asterisked.
[a] Expressed as mean (range).

related to the control of metabolism (Portillo et al., 1996). Thus, an extensive analysis of the cytoarchitecture and the relative location of HAP and LCA neurons should increase our knowledge of the intrahypothalamic network of the PVN related to the control of metabolism and feeding behaviour.

Two main observations were made in our work: firstly, there was a clear segregation, within the PVN, of HAP neurons from LCA neurons. Secondly, there was a significant presence, in the parvocellular dorsal subnucleus of the PVN, of FG/Fos double-labelled neurons, i.e. hypothalamospinal LiCl-activated neurons.

The distribution of HAP neurons observed in our study is in keeping with previous reports (Swanson et al., 1980; Portillo et al., 1996). These nerve cells surrounded LCA neurons at the levels where the two populations overlapped. The distribution of magnocellular LCA neurons described in the present report suggests that these cells are neurohypophyseal. In agreement with this view is the fact that plasma levels

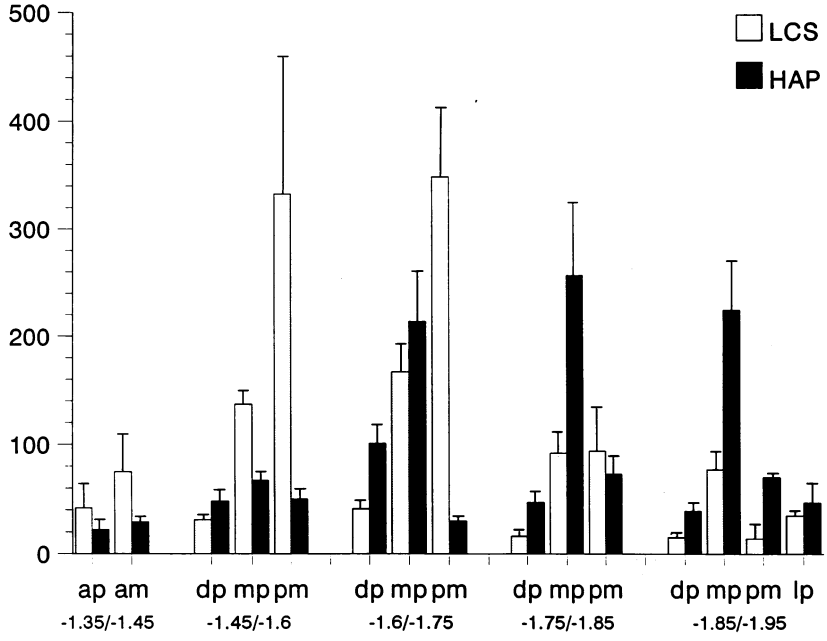

Fig. 4. Mean (±S.E.M.) numbers of hypothalamic fluorescent-labelled neurons which project to the injected autonomic preganglionic levels (HAP) and c-Fos-stained neurons after LiCl administration (LCS). The figure shows the different subnuclei of the PVN of the stimulated rats, at the levels where the two neuron populations overlapped. Numbers indicate (mm) the rostrocaudal position of the sections, zero being the bregma. ap, anterior parvocellular subnucleus of the PVN; dp, dorsal parvocellular subnucleus of the PVN; mp, medial parvocellular subnucleus of the PVN; lp, lateral parvocellular subnucleus of the PVN; am, anterior magnocellular subnucleus of the PVN; pm, posterior magnocellular subnucleus of the PVN.

of oxytocin increase after LiCl administration (McCann et al., 1989). Oxytocinergic and vasopressinergic neurons have been described in the posterior magnocellular subnucleus of the PVN (Rhodes et al., 1981; Giovannelli et al., 1990; Griffond et al., 1994). In addition, the location of the magnocellular LCA neurons coincides with the pattern of vasopressinergic neurons described in previous reports (Sawchenko and Swanson, 1982). The weak expression of c-Fos in supraoptic and PVN nuclei in control rats is in keeping with the hypothesis that the expression obtained after LiCl administration is not due to hypertonicity of the injected solution.

The presence of FG/Fos double-labelled cells in the dorsal parvocellular subnucleus of the PVN indicates that some LCA neurons within the PVN project to thoracic spinal sympathetic preganglionic levels, such as the ICC. This result is in agreement with the notion that some neurons of the dorsal parvocellular subnucleus of the PVN could mediate a nervous response after LiCl administration through the thoracic sympathetic preganglionic route.

On the other hand, the absence of significant differences in FB/Fos double-labelled neurons between controls and stimulated animals, is not compatible with the hypothesis that LCA neurons within the PVN project to vagal parasympathetic preganglionic levels, such as the DMX.

Further studies are needed to clarify whether LiCl-sensitive neurons project to other levels of the central nervous system related to feeding behaviour and metabolism, such as the parabrachial nucleus in the pons or periaqueductal grey in the midbrain (Swank et al., 1995; Bray, 1991).

In summary, within the PVN, LiCl-sensitive neurons are located in its rostral portion, mainly in the magnocellular division, while the neurons which project to autonomic preganglionic levels are present throughout the rostrocaudal extension, predominantly in the caudal two-thirds of the PVN. The presence of FG/Fos double-labelled cells shows that the parvocellular dorsal subnucleus of the PVN could mediate the thoracic sympathetic preganglionic route, being part of the efferent pathway involved in the responses observed after LiCl administration. Taken together, these data contribute additional knowledge of the neural hypothalamic network involved in the autonomic control of feeding.

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