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Coaxiality of Foxb1- and parvalbumin-expressing neurons in the lateral hypothalamic PV1-nucleus

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HIGHLIGHTS

- Foxb1-expressing neurons lie in the lateral hypothalamic PV1-nucleus.
- Coaxial distribution of Foxb1- and PV-expressing neurons in the PV1-nucleus.
- Foxb1-expressing neurons outnumber PV-expressing ones in the PV1-nucleus.
- Only a small proportion of the two neural populations co-express both markers.

In the ventrolateral hypothalamus, the PV1-nucleus is defined by its population of parvalbumin-expressing neurons. During embryogenesis, the ventrolateral hypothalamus is colonized also by Foxb1-expressing neurons. In adult *Foxb1-EGFP* mice, many immunofluorescent neurons were found within the region that is occupied by the PV1-nucleus. They formed a cloud around the axial cord of the parvalbumin-immunopositive cells, which they greatly outnumber (3:1). Only a small proportion of the neurons in the PV1-nucleus co-expressed both parvalbumin and Foxb1. In the light of these findings, a redesignation of this lateral hypothalamic structure as the PV1-Foxb1 nucleus would more accurately reflect its specific biochemical properties.

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1. Introduction

Progress in the understanding of the lateral hypothalamic functions has been hampered by the scarcity of markers for the tagging of its specific neuronal populations. We have previously described a novel lateral hypothalamic entity, the PV1-nucleus [1,2], which is defined by its population of parvalbumin-expressing neurons. It is lodged within the ventrolateral subarea of the lateral hypothalamus (LHVL) intermingled with the axons of the ventrolateral hypothalamic tract (vlt) of the medial forebrain bundle [3,4], disposed in the horizontal plane, and sandwiched between the optic tract laterally and the fornix medially [1,2].

Around embryonic day 14.5, Foxb1-expressing neurons stemming from the periventricular zone migrate towards the ventrolateral hypothalamus. *Foxb1* is a transcription factor and a member of the forkhead family of genes [5–9]. It was the aim of the present study to define more accurately the nature of the spatial relationship existing between Foxb1- and parvalbumin-expressing neurons in the region of the PV1-nucleus. Using adult *Foxb1-Cre-EGFP* mice, Foxb1-expressing neurons were revealed to form a sleeve around, and to greatly outnumber (3:1), the axially orientated parvalbumin-positive ones.

2. Materials and methods

2.1. Mice

Five adult Foxb1-Cre mice [8] were used in the present study. In this strain, Foxb1-expressing neurons co-express EGFP and

Abbreviations: f, fornix; LH, lateral hypothalamic area; LHVL, ventrolateral subarea of the lateral hypothalamic area; LM, lateral mammillary nucleus; MM, medial mammillary nucleus, medial part; MP, medial mammillary nucleus, posterior part; Opt, optic tract; PAG, periaqueductal grey; vlt, ventrolateral hypothalamic tract; 3v, third ventricle.

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Cre-recombinase. The animals were maintained on a 12-h light/12-h dark cycle at a constant temperature of 24 °C and fed *ad libitum*. The study was approved by the Cantonal Commission for Animal Experimentation (permit number: 201305FR).

2.2. Immunohistochemistry

After being anaesthetized (Pentobarbital; 20 μl/10 g per body weight), the mice were perfused first with physiological (0.9%) saline and then with phosphate (0.1 M, pH7.3)-buffered 4% paraformaldehyde. The brains were removed, immersed overnight in the same chemical fixative, and then transferred to a chilled (4 °C) 30% solution of phosphate (0.1 M)-buffered sucrose, for cryoprotection. Horizontal, 40 μm-thick cryosections were prepared using a freezing microtome (Frigomobil Reichert-Jung, Vienna, Austria) and collected in 0.1 M phosphate buffer (pH 7.3). Free-floating sections were exposed for 1 day at room temperature (RT) to a rabbit anti-EGFP antiserum [(Life Technologies, Japan, Ltd), diluted 1:1000 in TBS + 0.2% Triton-X 100 + 10% bovine serum]. The sections were rinsed in TBS and then exposed for 2 h at room temperature to the anti-rabbit biotinylated secondary antibody [(Vector Laboratories, Burlingame, CA, USA), diluted 1:200 in TBS + 10% bovine serum]. After rinsing once in TBS and twice in Tris–HCl (pH 8.2), the sections were exposed for 2 h to Alexa 488-conjugated streptavidin [(Jackson ImmunoResearch Laboratories, Inc., USA) diluted 1:200 in Tris–HCl (pH 8.2)]. All sections were then exposed for 1 day at room temperature to a primary monoclonal antibody against parvalbumin PV 235 [(Swant, Marly, Switzerland) diluted 1:1000 in TBS + 10% bovine serum] and then to an anti-murine Cy3 antibody [(Jackson ImmunoResearch Laboratories, Inc., USA) diluted 1:200 in Tris–HCl (pH 8.2)]. Cell nuclei were revealed by counterstaining with DAPI (diluted 1:2000 in phosphate-buffered saline) for 5 min at room temperature.

The sections were mounted and evaluated in either a Leica 6000 epifluorescence microscope [equipped with a Hamamatsu C4742-95 camera], a digital slide scanner (Nanozoomer, Hamamatsu)] or a Leica TCS SP5 confocal laser microscope.

Images were post-processed for brightness and contrast using Adobe Photoshop CS5. The image-stacks were prepared with ImageJ 1.44p software and the figures collated using the Adobe Illustrator CS6.

2.3. Cell counting

The Foxb1-EGFP- and the parvalbumin-immunopositive cells were counted using the optical fractionator method [10]. This task was executed with a Zeiss Photomicroscope (equipped with a Hamamatsu Orca 0G5 camera) using Stereoinvestigator 10.52 software (MBF.Bioscience). Counts were made on every second section using a 70 μm × 70 μm counting frame, which was placed at 100 μm intervals along the *X*- and *Y*-axes. The thickness of each section was set at 30 μm.

3. Results

In horizontal sections through the hypothalamus, parvalbumin-immunoreactive sites are limited to the lateral mammillary nucleus (LM), to a cluster of cells (the Circular nucleus) in the anterior region (not shown), and to the PV1-nucleus in the ventrolateral area (Fig. 1A). The PV1-nucleus is formed by a slender cord of parvalbumin-immunopositive neurons (boxed in Fig. 1A), which are intermingled with axons of the medial forebrain bundle. Since in the mice used in this study Foxb1-expressing neurons express also EGFP (Enhanced Green Fluorescent Protein) as a reporter, we used an antibody against EGFP to detect them. Foxb1-EGFP-immunopositive neurons are commonly encountered at the level

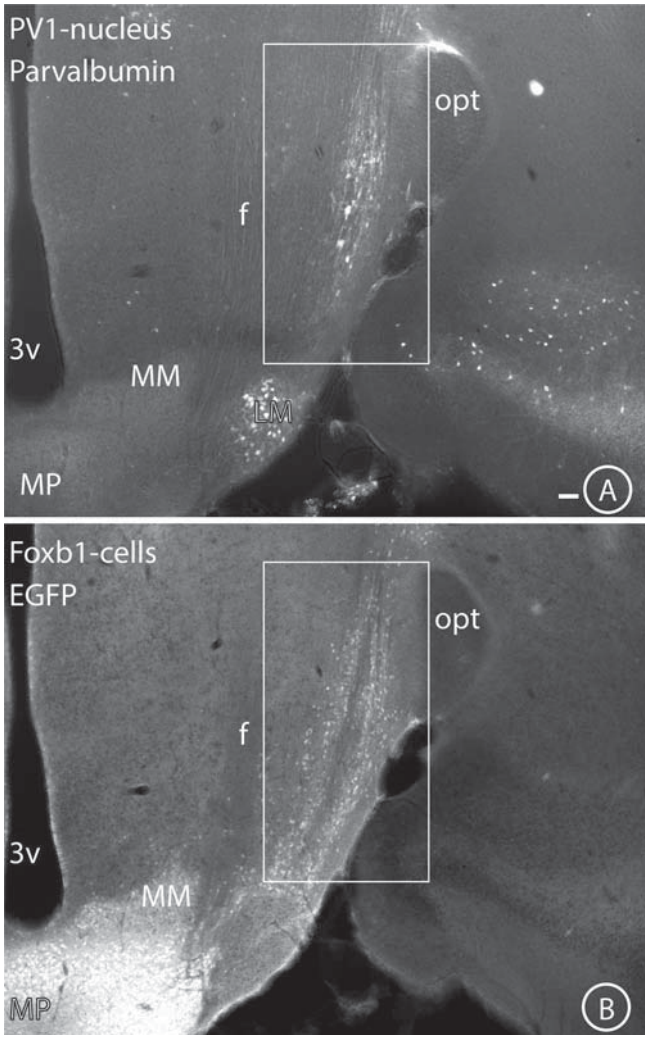

Fig. 1. Low-magnification images of the same horizontal section through the hypothalamus of a Foxb1-EGFP-expressing mouse revealing immunofluorescence for parvalbumin (A) and EGFP (B). (A) Parvalbumin-immunopositive neurons are encountered in the lateral mammillary nucleus (LM), and in the lateral hypothalamus, close to the optic tract, as well as in the PV1-nucleus (boxed). (B) Foxb1-EGFP-immunopositive neurons colonize nuclei of the mammillary complex (*e.g.*, MM and MP). Immunoreactivity is detected in single cells of the periventricular zone in the third ventricle and in a large contigent of neurons in the lateral hypothalamus, corresponding in location to the PV1-nucleus (boxed). Scale bar 50 μm.

of the mammillary bodies and in the lateral hypothalamic region (Fig. 1B). Ventrolaterally, Foxb1-expressing neurons form a broad, elongated structure, which is also located in the medial forebrain bundle. It extends from the interstitial nucleus of the stria medullaris to the medial mammillary nucleus, and is bordered by the optic tract laterally and by the fornix medially (Fig. 1B). In the region of the PV1-nucleus, Foxb1-expressing neurons are numerous; more numerous indeed than the PV-immunopositive ones (Fig. 1B).

Parvalbumin-immunopositive neurons are small and bipolar in the rostral portion of the PV1-nucleus, and large and multipolar in the caudal one [2], whereas Foxb1-expressing neurons are characterized by small, bipolar perikarya.

The results of the cell-counting analysis reveal the number of *Foxb1-EGFP*-expressing neurons to be 3-fold higher than that of the parvalbumin-expressing ones (Table 1). Hence, the parvalbumin-expressing cells of the eponymous nucleus are a minority population. Less than 10% of the total number of neurons

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Table 1
Mean total number of parvalbumin- and EGFP-expressing neurons in the lateral hypothalamus of adult mice, obtained by the optical fractionator method.

Marker	Region	Mean total number	Coefficient of error	Counting frame area (*XY*) (μm^2)	Sampling grid area (*XY*) (μm^2)
PV235	LH	290,55	$0.15 \leq CE \geq 0.29$	4900	10000
EGFP	LH	962,72	$0.14 \leq CE \geq 0.18$	4900	10000
Double labelling	LH	79,91	$0.18 \leq CE \geq 0.36$	4900	10000

The mean total number of parvalbumin (PV) and EGFP-immunopositive neurons, as well as the mean total number of cells that co-expressed the two markers, were estimated on sections through the lateral hypothalamus of 5 mice using the optical fractionator method. The precision of the numerical estimates is described by the coefficient of error, which embraces counting noise, systematic uniform random sampling and variances in section thickness.

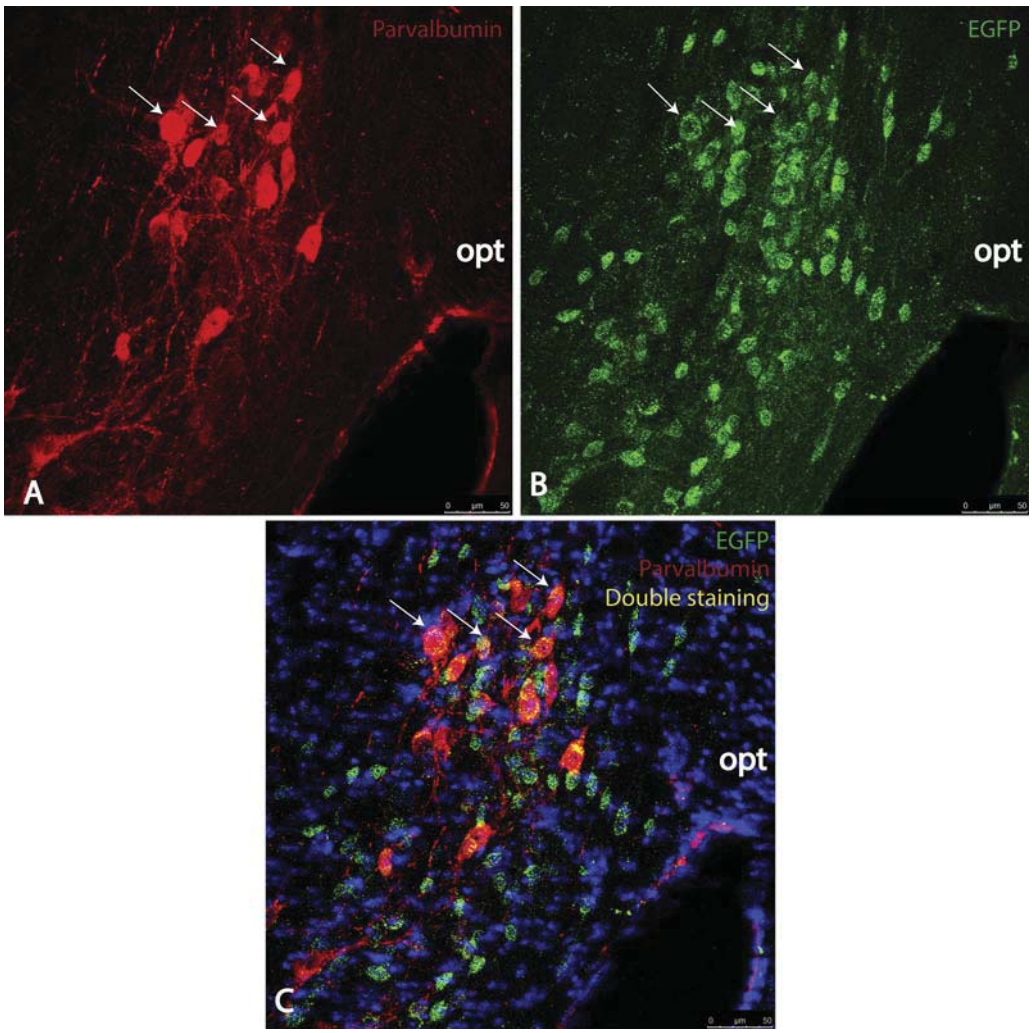

Fig. 2. High-magnification confocal images revealing Foxb1-EGFP- and parvalbumin-immunopositive neurons in a horizontal section through the lateral hypothalamus. (A) The cell bodies and the intermingling dendrites of the bi- and multipolar parvalbumin-immunopositive neurons are revealed in their completeness after exposure to a specific antibody. (B) Only the nucleus and perinuclear region of the neurons is granularly stained with the antibody against EGFP in this Foxb1-EGFP-expressing mouse. The contours of the cells themselves are not visible. (C) In a few cases, neurons expressing both EGFP and parvalbumin are encountered, as revealed after the merger of the images in A and B (arrows).

manifest double staining for both parvalbumin and Foxb1-EGFP (Table 1 and Fig. 2).

4. Discussion

Given the functional importance of the lateral hypothalamus and the dearth of specific markers for this region, the discovery of novel identifiable neuronal populations therein is to be heralded with interest. Within the adult ventrolateral hypothalamus, the location of the Foxb1-expressing population of neurons [11] coincides with that occupied by the PV1-nucleus [1,2]. This locus partially perpetrates the LHVL2-region, as defined in a seminal publication by Nieuwenhuys and his co-workers [3]. On the basis of our observation that a compact and well-defined mantle of Foxb1-expressing cells surround the parvalbumin-expressing neurons of the eponymous nucleus [1,2], we propose that this be henceforth referred to as the PV1-Foxb1-nucleus.

Our data reveal that the *Foxb1*-promoter remains active even after the completion of embryogenesis, insofar as the *Foxb1*-driven expression of *EGFP* was detected in the adult mice that comprised our study population. The pattern of *Foxb1-EGFP*-expression in the adult murine diencephalon corresponds to that of *Foxb1*-expression during embryogenesis [5]. The number of Foxb1-expressing neurons in this lateral hypothalamic region exceeds by a factor of

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3 that of the parvalbumin-expressing ones, albeit that in this murine strain, the latter are 2-fold less numerous than in wild-type (C56bl/6) mice (unpublished data). The parvalbumin-expressing neurons constitute the axis of the PV1-nucleus, which is mantled by a group of Foxb1-expressing ones, thereby giving the structure an appearance of coaxiality.

A small proportion of the Foxb1-immunoreactive neurons co-express parvalbumin, thereby implying that these cells stem from the caudal periventricular zone [11]. The origin of the other parvalbumin-expressing cells has not as yet been elucidated. It is conceivable that the PV1-Foxb1 nucleus is, in its entirety, built up of cells that have migrated from the lateral mammillary region, but that some of these later lose their capacity to express Foxb1.

The parvalbumin-immunopositive neurons of the PV1-nucleus project to a circumscribed longitudinal column of neurons, which is located in the midbrain, ventral to the aqueduct [12]. The question that now arises is whether the much larger population of Foxb1-EGFP-positive neurons likewise projects to this column, or to a functionally adjoining one in the PAG. To date, neither the inputs to the parvalbumin-expressing neurons nor those to the Foxb1-expressing ones have been identified.

The availability of Foxb1-Cre and parvalbumin-Cre mice will permit not only the targeted application of tracers and a definition of the connections of the two populations of neurons, but also the performance of specific ablation and activation experiments.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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References

[1] M.R. Celio, Calbindin D-28k and parvalbumin in the rat nervous system, Neuroscience 35 (2) (1990) 375–475.
[2] Z. Meszar, F. Girard, C.B. Saper, M.R. Celio, The lateral hypothalamic parvalbumin-immunoreactive (PV1) nucleus in rodents, J. Comp. Neurol. 520 (4) (2012) 798–815.
[3] L.M. Geeraedts, R. Nieuwenhuys, J.G. Veening, Medial forebrain bundle of the rat: IV. Cytoarchitecture of the caudal (lateral hypothalamic) part of the medial forebrain bundle bed nucleus, J. Comp. Neurol. 294 (4) (1990) 537–568.
[4] L.W. Swanson, Brain Maps III: Structure of the Rat Brain: An Atlas with Printed and Electronic Templates for Data, Models, and Schematics, 3rd rev. ed., Elsevier/Academic Press, Amsterdam/Boston, 2004, p. 215, 1 folded leaf of plates.
[5] G. Alvarez-Bolado, F. Cecconi, R. Wehr, P. Gruss, The fork head transcription factor Fkh5/Mf3 is a developmental marker gene for superior colliculus layers and derivatives of the hindbrain somatic afferent zone, Brain Res. Dev. Brain Res. 112 (2) (1999) 205–215.
[6] S.L. Ang, A. Wierda, D. Wong, K.A. Stevens, S. Cascio, J. Rossant, K.S. Zaret, The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins, Development 119 (4) (1993) 1301–1315.
[7] K.H. Kaestner, K.H. Lee, J. Schlöndorff, H. Hiemisch, A.P. Monaghan, G. Schütz, Six members of the mouse forkhead gene family are developmentally regulated, Proc. Natl. Acad. Sci. U.S.A. 90 (16) (1993) 7628–7631.
[8] T. Zhao, X. Zhou, N. Szabó, M. Leitges, G. Alvarez-Bolado, Foxb1-driven Cre expression in somites and the neuroepithelium of diencephalon, brainstem, and spinal cord, Genesis 45 (12) (2007) 781–787.
[9] G. Alvarez-Bolado, X. Zhou, A.K. Voss, T. Thomas, P. Gruss, Winged helix transcription factor Foxb1 is essential for access of mammillothalamic axons to the thalamus, Development 127 (5) (2000) 1029–1038.
[10] M.J. West, L. Slomianka, H.J. Gundersen, Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator, Anat. Rec. 231 (4) (1991) 482–497.
[11] G. Alvarez-Bolado, X. Zhou, F. Cecconi, P. Gruss, Expression of Foxb1 reveals two strategies for the formation of nuclei in the developing ventral diencephalon, Dev. Neurosci. 22 (3) (2000) 197–206.
[12] M.R. Celio, A. Babalian, Q.H. Ha, S. Eichenberger, L. Clément, C. Marti, C.B. Saper, Efferent connections of the parvalbumin-positive (PV1) nucleus in the lateral hypothalamus of rodents, J. Comp. Neurol. 521 (14) (2013) 3133–3153.

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