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The Dorsal Tegmentum of the Pontomesencephalic Junction of the Rat – Immunohistochemistry (Choline Acetyltransferase, Tyrosine Hydroxylase, Substance P) and NADPH-diaphorase Histochemistry in Frontal and Horizontal Sections

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With 4 Figures

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Summary: 37 complete frontal and horizontal series of rat brain were studied to compare the distribution of choline acetyltransferase- (ChAT), tyrosine hydroxylase- (TH), substance P- (SP), calbindin D- (Calb) and NADPH-diaphorase (NADPH-d)-positive cells within the cytoarchitectonic borders of the latero-dorsal tegmental nucleus (L-D) and its neighbourhood. We found the same distribution, number and morphology of NADPH-d-positive cells and ChAT-positive cells. Rostrally, there are no borders between NADPH-d-positive cells of L-D and NADPH-d-positive cells of the lateral part of the dorsal raphe nucleus. Only a few TH-positive cells are intermingled with ChAT/NADPH-d-positive cells at the lateral border of L-D. TH-positive cells are larger or the same size as cholinergic neurons. Locus coeruleus and its rostral part is full of TH-positive cells and their fibres run ventromedially towards L-D. Barrington's nucleus appears in double staining (ChAT and TH or NADPH-d and TH) as an empty area bordered by ChAT- or NADPH-d-positive cells of L-D and TH-positive fibres of the locus coeruleus. Some of these fibres run through the Barrington's nucleus. The shape and size of SP-positive neurons is the same as ChAT- and NADPH-d-positive neurons. SP-positive neurons are sparsely distributed in all parts of L-D, but there are only a few SP-positive cells in its medial part. About 50% of the ChAT- and NADPH-d-positive cells are also SP-positive.

Results are expressed by figures in three representative frontal sections and one horizontal section through the dorsal mesopontine tegmentum.

Key words: latero-dorsal tegmental nucleus, choline acetyltransferase, tyrosine hydroxylase, substance P, NADPH-diaphorase

Introduction

The latero-dorsal tegmental nucleus of Castaldi (L-D) (Castaldi, 1926) is located in the substantia grisea centralis (SGC) in the dorsal tegmentum of the pontomesencephaliç junction. It is bordered medially by the dorsal tegmental nucleus of Gudden (dG) and more rostrally by the dorsal raphe nucleus (RD), and laterally by the locus coeruleus (Coe). L-D lies under the surface of the fourth ventricle and its ventral part leaves the SGC in the ventrolateral direction. The caudal part of L-D contains a group of morphologically, histochemically and immunohistochemically different cells – Barrington's nucleus (Bar) (Barrington, 1925).

L-D plays an important role in the ascending reticular activating system (Moruzzi and Magoun, 1949), paradoxical sleep (Gnad and Pegram, 1980; Hobson et al., 1986; Sakai, 1988; Shiromani and Gillin, 1987; Kayama et al., 1992; McCarley et al., 1995), global attentive state in response to a novel stimulus (Koyama et al., 1994), regulation of regional blood flow in the thalamus (Koyama et al. (1994), regulation of the seizure treshold (Miller et al., 1991), oculomotor mechanisms (Cornwall et al., 1990), and release of acetylcholine in the basal forebrain (Prast and Philippu, 1992). Barrington's nucleus is involved in the micturition reflex (Barrington, 1925) and in stress (Imaki et al., 1991).

Cells of the L-D are immunohistochemically heterogenous, mostly known as cholinergic group Ch6 (Mesulam et al., 1983). Sutin and Jacobowitz (1988) described the distribution of cells positive for acetylcholinesterase (AChE), choline acetyltransferase (ChAT), galanin, neurotensin, substance P (SP), dynorphin B, vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), corticotropin-releasing factor (CRF) and atrial natriuretic factor (ANF) in the area of L-D. In L-D also the substances are present: ChAT mRNA (Oh et al., 1992), nitric oxide synthase (NOS) and ChAT (Dawson et al., 1991; Dun et al. 1994; Rodrigo et al., 1994), ChAT and SP (Vincent, 1983b), ChAT, SP and ANF (Skofitsch et al. 1985; Standaert, 1986), CRF (OLSCHOWKA et al., 1982; CUMMINGS et al., 1983; SWANSON et al., 1983; MERCHENHALTER, 1984; SAKANAKA et al., 1987), CRF mRNA (AUSTIN et al., 1995), CRF, SP and AChE (CRAWLEY et al., 1985), arginine succinate synthetase (NAKAMURA et al., 1991), citrulline (PASQUALOTTO et al., 1991), Calbindin D (GEULA et al., 1993), and GABA (FORD et al., 1995).

Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity was found in all cholinergic cells of L-D (VINCENT et al., 1983a; NAKAMURA et al., 1988; MESULAM et al., 1989; GEULA, et al., 1993). Topography of the cells and borders of the nuclei were similar to cresylviolet staining (PETROVICKÝ and NĚMCOVÁ, 1995), but NADPH-d-positive cells were also present in neighbouring structures. We divided the L-D into 4 parts (pars rostralis, pars medialis, pars centralis and pars ventralis). The present study was undertaken to compare the distribution of choline acetyltransferase-(ChAT), tyrosine hydroxylase- (TH), substance P- and NADPH-d-positive cells within the cytoarchitectonic borders of the L-D and its neighbourhood. This small immunohistochemical atlas of frontal and horizontal sections through the L-D will serve for future tract-tracing studies

Material and Methods

For this study the brains of 17 adult Wistar rats were used. The weight of the animals was 300g. The rat brains were treated with antibodies for choline acetyltransferase (ChAT, Chemicon, U.S.A.), tyrosine hydroxylase (TH, Incstar,U.S.A.), substance P (SP, Dr. Benoit, Montreal) and calbindin D_{28K} (Calb, Sigma, U.S.A.) and compared with 20 frontal series stained with cresylviolet or with NADPH-d rich neurons demonstration.

Immunohistochemistry: Animals were anesthetized with Narcovet, briefly perfused with Ca^{++}free Tyrode buffer (carbogenated) at pH 7,3 and perfused with 4% paraformaldehyde in phosphate buffer (PBS) at pH 7,3. After dissection, brains were stored in the same fixative solution overnight at 4°C. 75μm frontal vibratome sections were obtained and collected in 0,1 M phosphate buffer at pH 7,3 (PBS). Free-floating sections were washed several times in PBS and then preincubated for 1 hour at room temperature in a mixture of 0,1M PBS pH 7,3, 0,5% Triton-X-100 (Sigma,U.S.A.) and 0,1% bovine serum albumin (for ChAT and TH) or 0,1M PBS pH 7,3, 0,5% Triton-X-100, 0,1% bovine serum albumin (Sigma, U.S.A.) and 5% normal goat serum (for SP). Next, the sections were incubated overnight at room temperature in the primary antibody (diluted 1:500 for ChAT, 1:2,000 for TH and 1:20,000 for SP). After two washes, 30 min each in 0,1M PBS pH 7,3 at room temperature, the sections were immersed for 90 min in rabbit anti-mouse peroxidase (RAMPO, DAKO A/S, Denmark) diluted 1:200 for ChAT and TH, or in goat anti-rabbit immunoglobulin (Nordic, The Netherlands) 1:200 for SP. After two rinses, 30 min each in 0,1M PBS pH 7,3 at room temperature, the sections for demonstration of ChAT and TH were immersed for 10 min at room temperature in a solution containing 20 mg diaminobenzidine (DAB) and 300 mg Ni-ammonium sulphate in 100ml 0,05M Tris buffer at pH 7,6, followed by 5-10 min treatment with the same solution to which 10μl 30% H_2O_2 100 ml was added. The reaction was stopped by rinsing three times for 5 min in PBS 0,1M pH 7,3 at room temperature. The sections for demonstration of SP were, following two rinses after incubation with secondary antibody, immersed for two hours in a mixture of one drop of avidin, one drop of biotin complex in 100ml of PBS (ABC). This mixture must be prepared 30 min before use. After two rinses, preincubation and incubation were the same in the DAB-solution as in ChAT demonstration. DAB-solution without Ni-ammonium sulphate was used only for TH demonstration. Sections were mounted on slides coated with gelatin chrome-alum, air-dried, dehydrated, cleared in xylene and coverslipped with Entellan.

Horizontal 50 μm Vibratome sections were used for demonstration of ChAT, TH, SP, Calb and for double-labelling for ChAT and TH or NADPH and TH (freezing microtome sections). Preincubation was 60 min in a mixture of 0,1 M PBS pH7,3, 0,1% bovine serum albumin and 0,5% Triton X-100, incubation overnight at room temperature with mouse antibody anti ChAT 1:1,000, or mouse antibody anti TH 1:2,000 or rabbit antibody anti SP (from Dr. Benoit, Montreal) 1: 20,000 or mouse antibody anti Calb 1:5,000. After two rinses there was a 60 min. incubation with secondary antibody - donkey anti mouse biotin 1:1 500 for ChAT, TH and Calb, and donkey anti rabbit biotin 1:1 500 (for SP). After two washes the sections were incubated for 120 min. in ABC. Preincubation and incubation in the DAB-Ni solution were the same as above. Sections of three brains were rinsed two times after the demonstration of ChAT and incubated overnight at room temperature with the antibody anti TH. Incubations with secondary antibody and ABC were the same as above, only DAB-solution was used without Ni-ammonium sulphate.

NADPH-d histochemistry: For the demonstration of NADPH-d activity the SHERER-SINGLER et al. (1983) method was used, for the free-floating sections half of the concentrations of tetrazolium dye, beta-NADPH and sodium malate were enough. Every second section was stained with cresylviolet. Horizontal sections of one brain were used for demonstration of TH after NADPH-d demonstration .

As in our description of the topographical organization of NADPH-d positive neurons in L-D, we distinguish (PETROVICKÝ and NĚMCOVÁ, 1995) 4 parts of L-D in our series: From the main part (the pars centralis) arise the pars medialis, the pars ventralis and the pars rostralis.

The distribution of cells of the L-D in the different stainings was compared mainly on level I (Barrington's nucleus), level II (the transition of the fourth ventricle into the aquaeductus mesencephali) and on level III (the commissura colliculorum inferiorum) in frontal series and on level I-H (decussatio of the brachium conjunctivum), II-H (Barrington's nucleus), III-H (the IIIth. and IVth. cranial nerve nuclei) and IV-H (dorsal to the IIIth. and IVth. cranial nerve nuclei) in horizontal series.

Results

Choline acetyltransferase (ChAT)

The most caudal ChAT-positive neurons within the cytoarchitectonically defined borders of L-D were found at the level of the rostral part of the nucleus motorius nervi trigemini (Fig. 2a). They are located in the substantia grisea centralis medially from unstained cells of the nucleus mesencephalicus nervi trigemini (n.V.mes) and the locus coeruleus and lateral to the pale place corresponding with the position of the dorsal Guddens tegmental nucleus (dG). Some of the ChAT-positive neurons are shifted laterally from the main constellation of L-D by the unstained Barrington's nucleus. Some ChAT-positive cells are spread tightly under the surface of the fourth ventricle and also among the fibres of the ventral part of the mesencephalic trigeminal tract.

More rostrally, the "pars medialis" arises from the central part of the L-D above the dorsal surface of the unstained dorsal Guddens tegmental nucleus (Figs. 2 b,c, "Level I and II" on Fig. 1).

On the level of transition of the fourth ventricle into the aquaeductus mesencephali, the large "pars ventralis" runs from the main constellation of the L-D, leaves SGC, and continues towards the brachia conjunctiva (Figs.2 b,c, "Level II" on Fig. 1). The cells of this part are sparsely distributed but by their shape and size are not different from cells in the central part of L-D. More rostrally this processus of cholinergic cells enters the cytoarchitectonic borders of the nucleus parabrachialis.

On the level of commissura colliculorum inferiorum the central part of the L-D is found more medially and the narrow strip of ChAT-positive cells ("pars rostralis") arises laterally. These cells are intermingled with ChAT-positive cells of the parabrachial nucleus and more rostrally with cells of the nucleus pedunculopontinus (Ch5, MESULAM, 1983). They constitute an irregulary garland of ChAT-positive cells around the brachia conjunctiva (Fig. 2e, "level III" on Fig.1). There are fewer and fewer ChAT-positive neurons in the substantia grisea centralis on more rostral levels. On the level of the nucleus nervi trochlearis they were present only outside the substantia grisea centralis – in the nucleus pedunculopontinus.

Using ChAT immunohistochemistry we could recognize middle-sized (20-30 μm), multipolar neurons with a slightly pale appearance of slightly eccentricly placed nuclei in L-D (Fig. 2c). Cells are not only multipolar but also oval, triangular, drop-like, elongated spindle or elongated with unregulary widenings. Long processes of neurons can be waved but mostly they are straight, dichotomously divided. In the rostral part of L-D (comparing to its other parts) there are more elongated cells.

Substance P (SP)

The most caudal SP-positive cells within the cytoarchitectonic borders of L-D were found on the level where the sulcus medianus is still deep on the floor of the fourth ventricle. Laterally from poorly-stainable small cells of the pars dorsalis of the dorsal Guddens tegmental nucleus are well-stainable middle-sized neurons of the caudal part of the L-D. Some of these neurons are focused also laterally from the unstained Barrington's nucleus.

The lateral part of the substantia grisea centralis between the mesencephalic trigeminal tract and the medial group of neurons of L-D is also full of SP-positive fibres in contrast to little SP-positivity in the area of the dorsal Guddens tegmental nucleus (Fig. 2f). Even greater density of SP-positive fibres is found in the nucleus parabrachialis and in the area of "nucleus sphenoidalis" (PAXINOS and BUTCHER, 1985) or "nucleus incertus" (SUTIN and JACOBOWITZ, 1988).

More rostrally (like in ChAT immunohistochemistry) from the main part of L-D, the "pars medialis" arises dorsally above the unstained dorsal Guddens tegmental nucleus and the "pars ventralis" procee-des into the parabrachial area. We found relatively few SP-positive cells in the medial part of the L-D as compared to ChAT-positive cells ("Levels I and II" in Fig.1). SP-positive fibres fill the area of the dorsal raphe nucleus and there are also some small cells under the bottom of the fourth ventricle.

On the level of the commissura colliculorum inferiorum SP-positive cells are less numerous in the substantia grisea centralis, and most of them are focused around the brachia conjunctiva, in the area of the nucleus pedunculopontinus, similar to the ChAT-positive cells. More rostrally, on the level of the nucleus interpeduncularis, L-D disappears and SP-positive cells are sparsely distributed in the substantia grisea centralis.

SP-positive cells in the cytoarchitectonic borders of the L-D are middle-sized multipolar, triangular, oval, drop-like or elongated cells with smooth or beaded fibres dichotomously divided in different distances from the cell's body. Their shapes are not different from those of ChAT-positive neurons in the L-D area, but there are half as many SP-positive neurons on "Level I and III" and one-third as many on "Level II."

Tyrosine hydroxylase (TH)

Cells within the cytoarchitectonic borders of L-D contain no tyrosine hydroxylase. But the laterally neighbouring locus coeruleus is composed of TH-positive noradrenergic neurons. Only few of its TH-positive cells are intermingled with ChAT-positive cells on the lateral margin of L-D. The larger part of the locus coeruleus is located more caudally than L-D. Locus coeruleus projections run medially in the

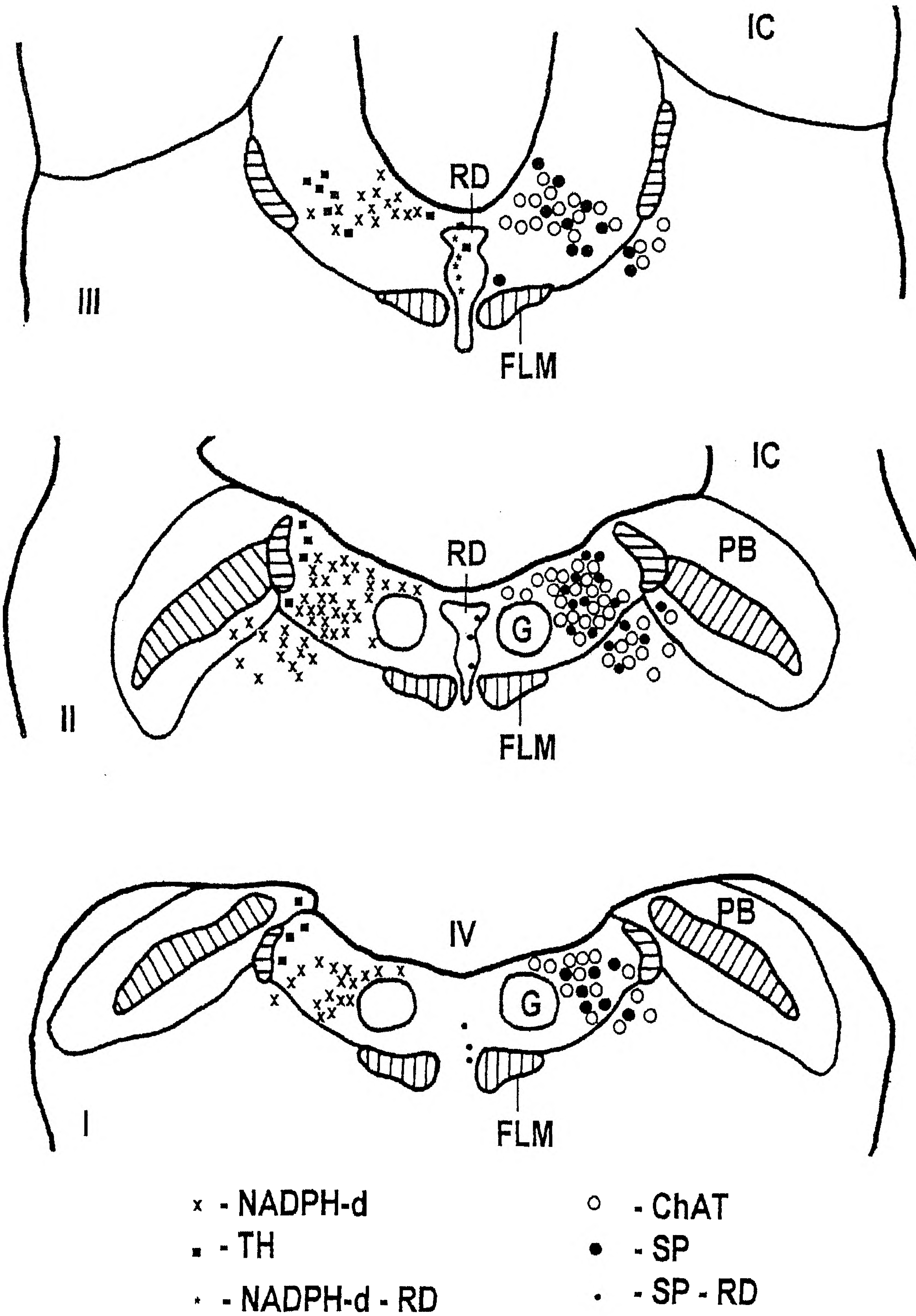

Fig. 1. Schematic representation of the relative distribution of NADPH-d-, TH-, ChAT- and SP-positive cells in L-D and its neighbourhood at three representative levels of frontal sections.
I - the most caudal level , III - the most rostral level, IV - IV. ventricle, FLM - fasciculus longitudinalis medialis, G - dorsal tegmental nucleus of Gudden, IC - colliculus inferior, PB - parabrachial nucleus, RD - dorsal raphe nucleus

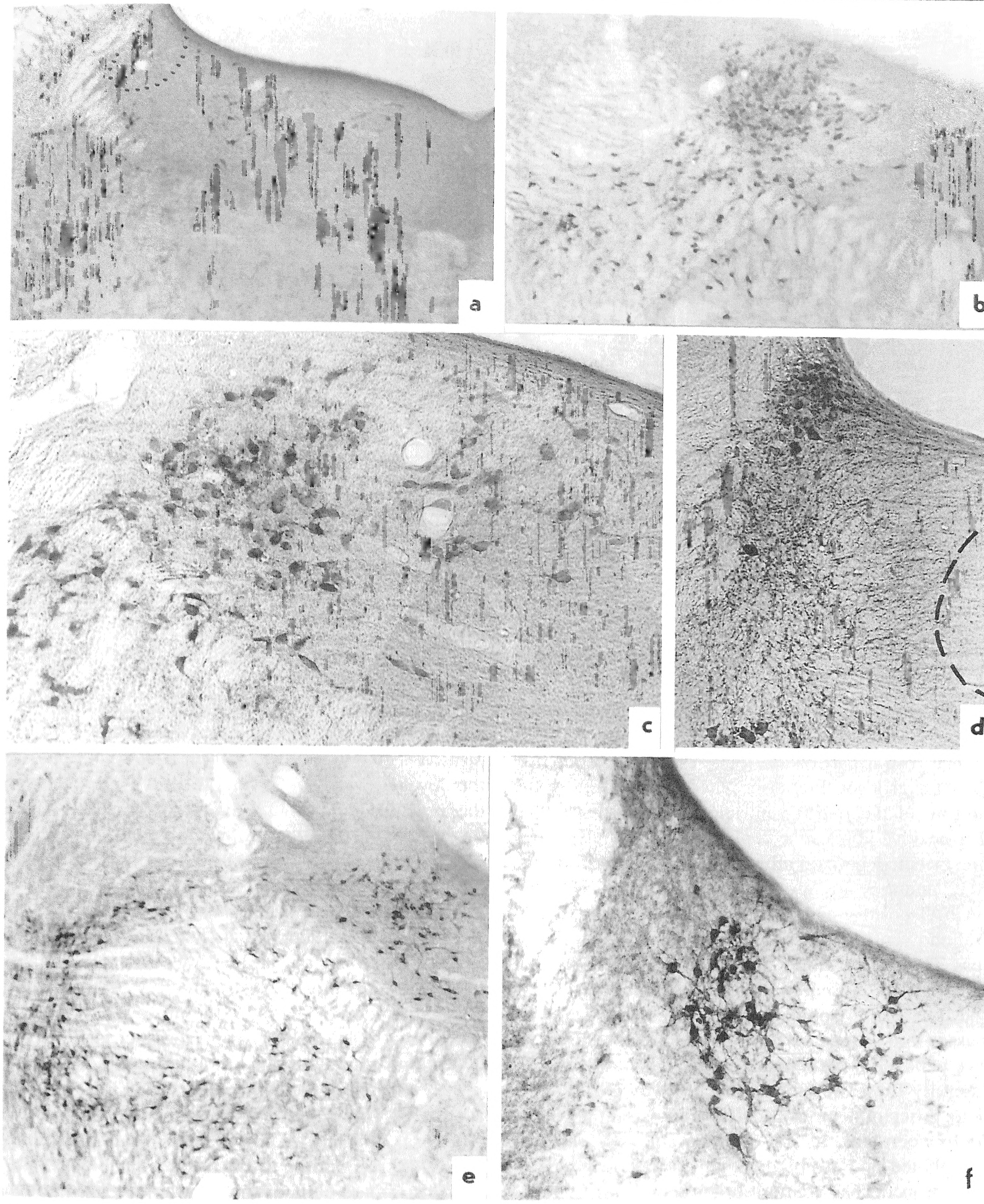

Fig. 2. Frontal sections at different levels of L-D stained for ChAT, TH or SP immunohistochemistry.
a - the caudalmost ChAT-positive cells on the caudal edge of L-D. The area of Barrington's nucleus contains no ChAT-positive cells. The dotted line shows the medial and caudal borders of unlabelled locus coeruleus.(Magnification 35x); **b** - ChAT-positive cells of L-D at the "level II". (Magnification 35x); **c** - ChAT-positive cells of the medial, central and ventral parts of L-D in higher magnification. ("level I -II").(Magnification 100x); **d** - TH+ cells and fibers of the locus coeruleus. Transverse fibres extend through the area of L-D but not through the area of the dorsal tegmental nucleus of Gudden (the interrupted line shows the lateral border of the dorsal tegmental nucleus of Gudden). (Magnification 100x); **e** - ChAT-positive cells in the rostral part of L-D and cells of Ch5 group, surrounding the brachium conjunctivum. (Magnification 35x); **f** - SP-positive cells of medial and central parts of the L-D. The locus coeruleus is full of SP-positive fibres. (Magnification 100x)

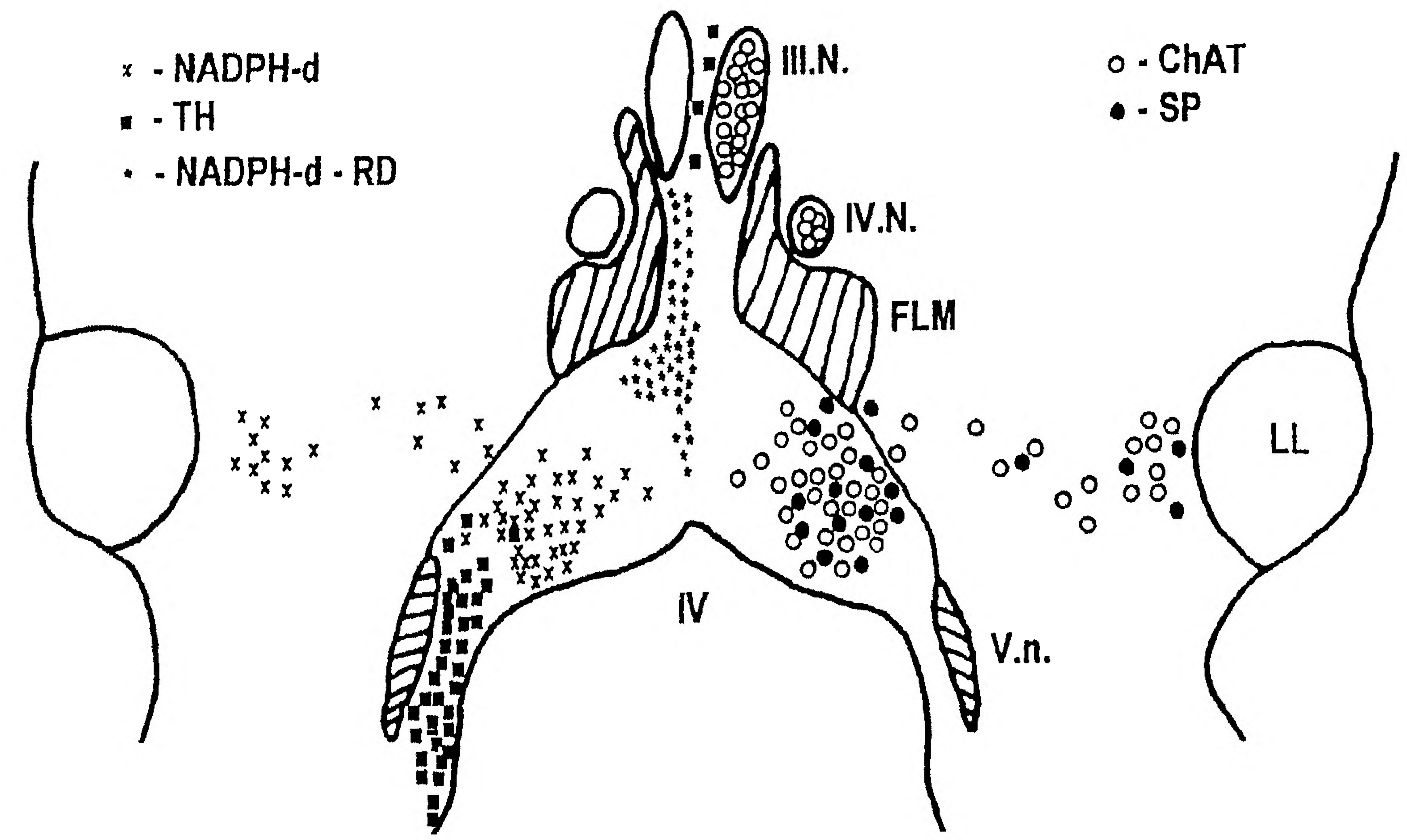

Fig. 3. Schematic representation of the relative distribution of NADPH-d-positive (crosses), TH-positive (squares), ChAT-positive (circles) and SP-positive (dots) cells in the area of L-D and its neighbourhood in horizontal section through the the oculomotor nucleus ("Level IIIH"). Little asterisks indicate NADPH-d-positive cells in the dorsal raphe nucleus. III.N - nucleus of the third nerve, IV.N. - nucleus of the fourth nerve, V.n. - mesencephalic trigeminal tract, IV - fourth ventricle, FLM - fasciculus longitudinalis medialis, LL - lateral lemniscus

substantia grisea centralis and ventrolaterally to the group A7 of TH-positive cells.

The rostral part of the locus coeruleus Coe (Fig. 2d, "Level I" on Fig 1) is placed tightly under the bottom of the fourth ventricle. Densely distributed TH-positive fibres arise ventrally and medially. They are oriented horizontally in the lateral half of the SGC and they are in close proximity with NADPH-d/ChAT-positive and SP-positive neurons in the central and ventral part of L-D. In Barrington's nucleus less TH-positive fibres are present. Some TH-positive cells are present in the parabrachial area.

More rostrally on the level where the IV. ventricle passes into the aquaeductus mesencephali, TH-positive cells lie in the substantia grisea centralis tightly laterally from ChAT- and SP-positive cells of L-D. This group of neurons is called the rostral part of the locus coeruleus. Its TH-positive cells are sparsely distributed and are bigger then in the locus coeruleus proper. They are multipolar neurons with 3 or 4 thicker processes, dichotomously divided in different distances from the cell's body.

ChAT – horizontal sections

The first part of L-D visible in horizontal sections, studied from ventral to dorsal levels, is its ventral part. ChAT-positive cells of this process continue to the pedunculopontine nucleus . There are no ChAT-positive cells in the substantia grisea centralis on this level (Level IH). On this level the decussatio of the brachium conjunctivum passes rostrally from the fasciculus longitudinalis medialis. Cholinergic cells run rostrocaudally from the rostral edge of the SGC and the lateral border of the ventral tegmental nucleus of Gudden through the bracha conjunctiva to the medial border of the lateral lemniscus. They are multipolar, sparse, and without common orientation. They are mostly rostrocaudally oriented only on medial edge of the lateral lemniscus.

On level II-H, dorsally from the decussatio of the brachium conjunctivum cholinergic neurons are mostly located in the central and medial part of L-D (ventrally from the dorsal tegmental nucleus of Gudden). There is an empty area in the lateral half of L-D corresponding to Barrington's nucleus. Sparsely distributed cells of the ventral part run through the fasciculus longitudinalis medialis and brachia conjunctiva to the group of ChAT-positive cells on the medial border of the lateral lemniscus.

On level III-H, in the area of the third and fourth cranial nerve nuclei, L-D looks like an oval nucleus with densely distributed cells without a common orientation in the substantia grisea centralis (Figs. 3, 4d). It is possible to distiguish the medial part of L-

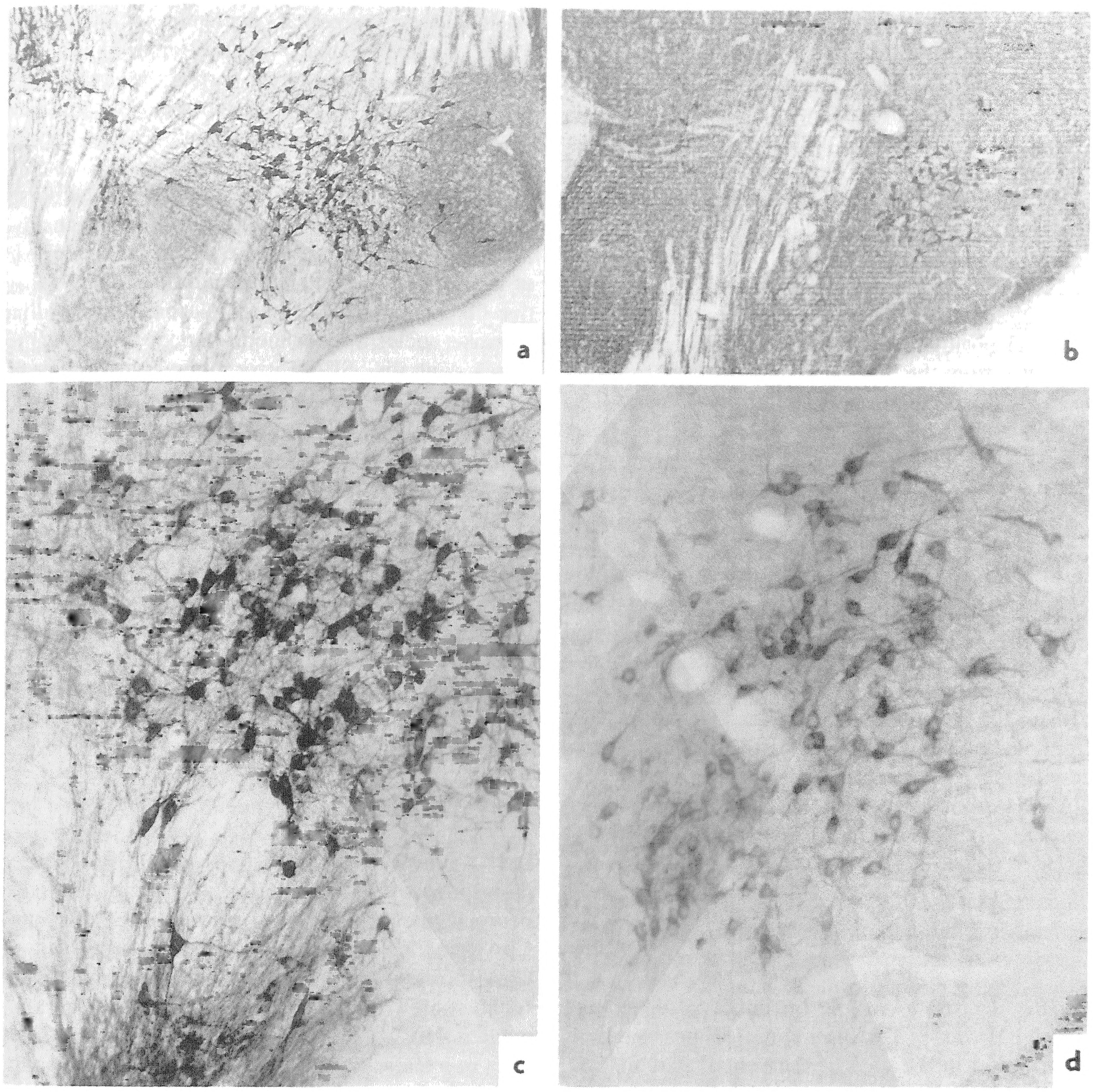

Fig. 4: Horizontal sections at different levels of L-D (NADPH-d histochemistry, ChAT or SP immunohistochemistry and double staining for NADPH-d and TH.
a - central, ventral and medial parts of L-D, empty area of Barrington's nucleus and locus coeruleus and oblique fibers connecting the L-D and the pedunculopontine nucleus. (NADPH-d staining, "level II-H"). (Magnification 40x); **b** - SP-positive cells in the central part of L-D and densely SP-positive fibres around brachia conjunctiva ("level III-H"). (Magnification 40x); **c** - NADPH-d-positive cells of central and medial parts of L-D and TH-positive fibers surround the empty area of Barrington's nucleus ("level II-H"). (Magnification 130x); **d** - ChAT-positive cells of central and medial parts of L-D ("level III-H"). (Magnification 130x)

D, with more sparsely distributed cells, from the central part, with a dense distribution of cells. The narrow strip of cells (the pars rostralis of L-D) begins from its rostrolateral edge and goes through pedunculi cerebellares superiores to the ChAT-positive cells of the parabrachial nucleus. Dorsally from the oculomotor nucleus (level IV-H) L-D ends as sparsely distributed cells in the SGC.

ChAT/TH – horizontal sections

In the double staining ChAT/TH the ventral part of L-D and the pedunculopontine nucleus is on level I-H. TH-positive cells of the locus coeruleus were found in the lateral part of the substantia grisea centralis. Locus coeruleus neurons are very densely distributed and their fibres run rostromedially in the substantia grisea centralis and enter the ventral part of L-D. Only a few large TH-positive neurons are intermingled with ChAT-positive neurons laterally, in the ventral part of L-D.

The same distribution of TH-positive cells is on level II-H. TH-positive locus coeruleus fibres with ChAT-positive cells surround the "empty" area of Barrington's nucleus.

In the more dorsal sections a few TH-positive cells were found among the ChAT-positive neurons of L-D. Some of them have the same shape and size as ChAT-positive neurons; some of them are larger. Laterally from the brachia conjunctiva and caudally from the cholinergic cells the group of large TH-positive cells is located near the lateral lemniscus.

NADPH-d/TH – horizontal sections

In the area of L-D, the shape, size and distribution of NADPH-d-positive cells is the same as in cholinergic neurons (Figs. 3, 4c). On levels III-H and IV-H (in the area of the III.nerves nucleus and above it) the group of smaller and less NADPH-d-stainable cells of the dorsal raphe nucleus is located medially from L-D. In NADPH-d histochemistry, fibres connecting the central part of the L-D with NADPH-d-positive group of cells on the medial border of the lateral lemniscus, are well shown (Fig. 4a).

Substance P – horizontal sections

On the decussatio of the brachium conjunctivum (level I-H), as in ChAT-staining, SP-positive cells were found in the area of the ventral part of L-D. These cells are without common orientation. They lay also in the fasciculus longitudinalis medialis and in the brachia conjunctiva and they are more densely distributed medially from the lateral lemniscus. An intensively SP-positive ventrodorsally-running narrow strip of fibres is located near the midline, caudally from the decussatio of the brachium conjunctivum. These fibres are present on more dorsal sections in the medial part of the fasciculus longitudinalis medialis.

On level II-H (the biggest cut of the fasciculus longitudinalis medialis), these fibres are located mediocaudally from the fasciculus longitudinalis medialis and they disappear on more dorsal sections. There are well-stainable, medium-sized neurons in the central and ventral part of the L-D. The medial part is developed only rostrally from the dorsal Guddens tegmental nucleus. The area of the dorsal Guddens tegmental nucleus is SP-negative. There are only small, densely-distributed light-stainable cells in the dorsal part of the dorsal Gudden's tegmental nucleus. The area of lenticular shape named "sphenoidal nucleus" (Paxinos and Butcher, 1985) or "nucleus incertus" (Sutin and Jacobowitz, 1988), on the caudal edge of the dorsal Gudden's tegmental nucleus, is full of SP-positive fibres. Cells in this area are only in the caudal part of the dorsal Gudden's tegmental nucleus. SP-positive fibres and small cells are placed in the midline in raphe nuclei. In the lateral part of the substantia grisea centralis, SP-positive fibres run craniocaudally around SP-negative cells of the mesencephalic trigeminal nucleus. The area between this nucleus and the lateral border of the central part of the L-D contains craniocaudally running SP-positive fibres but no SP-positive cells.

On levels III-H and IV-H the distribution of SP-positive cells is the same as that of ChAT-positive neurons (Figs. 3, 4b).

The only differences in all levels are SP-positive neurons being half in number and only a few SP-positive neurons being present in the medial part of the L-D.

Discussion

Sutin and Jacobowitz (1988) made a detailed description of the distribution of peptides and other neurochemicals in L-D and adjacent area. We found ChAT-positive neurons in the same areas but no ChAT-positive neurons in the locus coeruleus. They found some ChAT-positive neurons in Barrington's nucleus but we did not. We observed SP-positive cells in nucleus "O" and pars alpha SGC and in the raphe nuclei but in Barrington's nucleus we saw only a dense plexus of SP-positive fibres. Sutin and Jacobowitz (1988) did not describe less-stainable cells in the dorsal part of the dorsal part of the dorsal Gudden's tegmental nucleus. They only described a subgroup of well SP-positive fibres in a bundle in the dorsal and mediodorsal part of the dorsal Gudden's tegmental nucleus, for which they introduced the term nucleus incertus. They supposed colocalization of SP and ChAT in larger neurons of the L-D, which also by Vincent et al. (1983b) (30% of ChAT-positive cells contain SP) and by Standaert et al. (1986) (50% of ChAT-positive cells contain SP) has been indicated. Sutin and Jacobowitz (1988) supposed a neuromodulatory role of SP in cholinergic neurons of the L-D and in serotoninergic neurons of the dorsal raphe nucleus. Standaert et al. (1986) also found colocalization of SP, ANF and

CRF in cholinergic neurons of the L-D. In our sections very few SP-positive neurons were present in the medial part of the L-D and there were approximately half as many SP-positive cells compared to ChAT cells in the other parts of the L-D. Sutin and Jacobowitz found seven neuropeptides in the L-D. SP, galanin, CGRP and CRF show similar density and distribution within L-D - approximately the same pattern as ChAT-positive cells. Neurotensine-positive cells are present along the ventral border of L-D and they merge into the central tegmental tract. VIP-positive cells are few in number and remain confined to the ventrolateral corner of L-D and only a few cells along the lateral border of L-D express dynorphin.

Our maps of cholinergic neurons are in good agreement with those of MESULAM et al. (1983) and VINCENT et al. (1983a), who combined NADPH-d histochemistry with ChAT immunofluorescence and showed that only ChAT-positive neurons are stained by NADPH-d-histochemistry in the area of L-D. According to our findings the use of NADPH-d histochemistry is successful for detailed demonstration of Ch6 group neuron fibres.

DUN et al. (1994) and RODRIGO et al.(1994) studied NOS reactivity in the rat brain. In brainstem nuclei, they found the highest cNOS immunoreactivity in L-D and the pedunculopontine nucleus. Dunn described the size of NOS-positive neurons in L-D as 20-25 μm and a few cells as 2-7 μm. We did not observe such small cells in ChAT, SP or NADPH-d staining in the area of L-D. The size of neurons in our sections was 20-30 μm. RODRIGO et al. (1994) found some cNOS positive cells in the area of the locus coeruleus, but neither DUNN et al. (1994), using cNOS immunohistochemistry, nor VINCENT et al. (1983), NAKAMURA et al. (1988), or GEULA et al. (1993), using NADPH-d histochemistry found positive cells in the locus coeruleus. Perhaps Rodrigo described cNOS-positive cells on the lateral border of Barrington's nucleus in the caudal part of L-D.

Barrington's nucleus and adjacent area were studied by RIZVI et al. (1994). Our frontal sections double-stained for NADPH-d and TH are similar. We found the most caudal ChAT-positive cells medially from Barrington's nucleus corresponding to L-D in Nissl staining. The most caudal NADPH-d-positive cells in the substantia grisea centralis of mesopontine tegmentum were found laterally from Barrington's nucleus but not within the cytoarchitectonically (Nissl staining) defined borders of L-D. Hence, we hesitate to add the most caudal NADPH-d positive cells to L-D. In contrast, RIZVI et al. (1994) determine them as cholinergic L-D neurons.

There was only a small overlap of ChAT/NADPHd-positive cells and TH-positive cells on the lateral border of the L-D and we did not observe in rats large intermingling of NADPH-d-positive and TH-positive cells in L-D as LEONARD et al. (1995) described in guinea pigs.

L-D contains not only medium-sized ChAT/NADPH-d-positive neurons but also small ChAT/NADPH-d-negative cells. GEULA et al. (1994) demonstrated no overlapping co-localization of small Calb-positive neurons with the cholinergic neurons of Ch5 and Ch6. FORD et al. (1995) even demonstrated approximately twice as many small ChATnegative/GABAergic neurons as ChAT-positive neurons in L-D. Perhaps there is some colocalization of GABA and calbindin in small cells of the L-D. Our preliminary results in the demonstration of Calb-positive neurons show similar but not identical distribution of calbindin and substance P in the ponto-mesencephalic tegmentum. On the level I-H (decussatio of the brachium conjunctivum) in horizontal sections we found large Calb-positive cells in the nucleus ruber, and medium-sized cells in the lateral lemniscus, medium-sized and small cells in the ventral and dorsal part of the nucleus parabrachialis, and a dense plexus of Calb-positive fibres in the medial part of the fasciculus longitudinalis medialis, in the dorsal part of the nucleus parabrachialis and in the ventral Gudden's tegmental nucleus. There are distributed fibres less densely in the area of the dorsal raphe nucleus, ventral part of the parabrachial nucleus, in the medial vestibular nucleus and in the area medially from the locus coeruleus. Calb-positive fibres surround Calb-negative cells of the mesencephalic trigemnal nucleus. Cells in the area of the L-D are small and a lot of such cells run to the fasciculus longitudinalis medialis.

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