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Catecholaminergic and dopamine-containing neurons in the spinal cord of pigeons: an immunohistochemical study

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Abstract

Within the different species belonging to the vertebrate radiation, catecholaminergic elements of the spinal cord present a partly conservative, partly variable pattern. Unfortunately, the overall picture is far from clear since the situation for birds is largely obscure. Therefore, we examined the distribution of dopamine (DA)- and tyrosine hydroxylase (TH)-positive cells and fibers in the spinal cord of the adult pigeon by immunohistochemistry. TH-immunoreactive cells were located within two restricted areas. One group of cells with multipolar shape was located in laminae VI and VII, close to the white–gray border. These cells were more frequently found at rostral and caudal levels while being scarce at cervical-thoracic levels. The second group of cells was located in lamina VIII surrounding the central canal. These cells were bipolar in shape and were found ventrally and laterally to the central canal, with most of them contacting the lumen of the canal through a separate process. The TH-immunoreactive fibers were distributed in both the gray and the white matter. In the gray matter, they were mainly distributed around the central canal (lamina VIII), in the ventral horn close to the border of laminae VII–IX and in the lateral part of the dorsal horn in laminae II–VI. In the white matter the fibers were present in the lateral columns running longitudinal to the main axis. DA-immunoreactive cells were also located within two restricted areas, closely matching the distribution of TH-immunopositive ones. Additionally, the DA-immunoreactive cells had the same shape as the TH-immunoreactive cells, as bipolar neurons contacted the central canal and multipolar ones were located in the laminae VI and VII. Also the distribution of DA- and TH-immunoreactive fibers roughly matched. Both, DA-immunoreactive cells and fibers were scarcer than TH-immunoreactive ones. This finding suggests that the catecholaminergic system in the spinal cord consists of DA-immunoreactive cells as well as other catecholaminergic cells.

Keywords: Tyrosine hydroxylase; Birds; Liquor-contacting-neurons; Motor pattern generator

1. Introduction

The spinal cord has a highly complex architecture with various functionally distinct cell groups that can be partly distinguished by their neurochemical diversity. One of these cellular systems are the spinal dopaminergic neurons which were described in cartilaginous and osseous fish, reptiles and different mammals (Stuesse et al., 1991; Roberts and Meredith, 1987). These studies revealed variations in the spinal dopaminergic system that are possibly related with different swimming or walking patterns created by the body trunk. Unfortunately, birds were rarely studied although they utilize a completely different style of locomotion and should therefore have attracted more interest. In avian species, mainly the distribution of tyrosine hydroxylase (TH) was examined (Guglielmone, 1995; Okado et al., 1991; Chikazawa et al., 1983). TH is the first enzyme of the

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catecholaminergic biosynthesis and catalyzes the reaction from L-tyrosine to L-DOPA. As such, it is of course not specific for dopamine (DA) but labels all catecholaminergic neurons. In the present investigation we have therefore used a monoclonal antibody against DA, in addition to a monoclonal antibody against TH.

Catecholaminergic cells and fibers of the non-mammalian spinal cord are mainly found in two locations. One group consists of neurons contacting the liquor of the central canal. These liquor-contacting-neurons (LCNs) are located ventrally to the central canal, are bipolar with an amphora shape and protrude with a single process into the ventricular zone (Roberts et al., 1995). The non-LCNs have more diverse locations which vary between different species. In lampreys, for example, there are two sub-populations. One of them consists of multipolar cell bodies located in the midline region below the central canal, the other consists of few neurons located within the lateral cell column (Pombal et al., 1997; Schotland et al., 1996). In teleosts and birds, non-liquor-contacting TH neurons have also been reported (Roberts et al., 1989; Okado et al., 1991; Wallace et al., 1987, 1996). In the European eel these multipolar catecholaminergic neurons are located in the dorsomedial portion of the spinal cord (Roberts et al., 1989). In the chicken, these cells occur in the superficial and lateral border of the dorsal horn (Wallace et al., 1996). Different from non-mammalian vertebrates, the catecholaminergic neurons in the spinal cord of rats are located in the dorsolateral half of the gray matter, and do not possess processes contacting the central canal (Mouchet et al., 1986). Taken together, it seems that catecholaminergic cells in the spinal cord present conservative features as well as highly variable elements. Catecholaminergic LCNs can be found in virtually all vertebrate classes except mammals, while the presence or absence as well as the location of TH-positive neurons in other spinal locations seem to vary to a large extent.

Although studies showing DA-immonureactive cells in the spinal cord of vertebrates are very limited, the available data point to similar locations as TH-immunoreactive neurons. In lampreys, some of the catecholaminergic LCNs as well as TH neurons in the ventrolateral and lateral cell columns of spinal cord were shown to be DA-immunoreactive (Pombal et al., 1997; Schotland et al., 1996). In the eel, multipolar dopaminergic neurons were found in the dorsomedial portion of the spinal cord (Roberts et al., 1989). In adult mammals, there is up to now no direct evidence of spinal dopaminergic neurons (Holstege et al., 1996; van Dijken et al., 1996; Mouchet et al., 1992).

Demonstrations of catecholaminergic cells in the spinal cord of birds were carried out in hatching birds only (Wallace et al., 1987, 1996; Guglielmone, 1995; Okado et al., 1991; Chikazawa et al., 1983). These studies found no direct evidence for the presence of dopaminergic neurons in the chicken, because DA could only be detected after addition of the DA precursor L-DOPA (Wallace et al., 1996). Based on this evidence it is not safely possible to argue that these DA-immunoreactive cells use DA as a neurotransmitter. Additionally, the utilization of premature animals may only demonstrate transient expressions of enzymes and transmitters. Therefore, the present study analyzes and documents the distribution of TH- as well as DA-immunoreactive cells in the spinal cord of the adult pigeon. We observed a profuse catecholaminergic innervation of the spinal cord, as previously demonstrated in hatching chickens (Wallace et al., 1987, 1996; Okado et al., 1991; Chikazawa et al., 1983). Additionally, the results suggest, that at least a part of the catecholaminergic neurons are of dopaminergic nature.

2. Method

2.1. Animals and tissue preparation

A total of nine adult pigeons (*Columba livia*) from local stock were used. Animals were injected with 1000 IU heparin 15 min prior to perfusion. Subsequently they were deeply anaesthetized with 0.3–0.4 ml Equithesin per 100 g body weight. For the TH-immunoreactivity study, six pigeons were perfused intracardially with 300 ml 0.9% saline (40 °C) followed by 1000 ml of fixative consisting of 4% paraformaldehyde in 0.12 M phosphate buffer (PB; 4 °C, pH 7.4). After perfusion, spinal cords were dissected and stored for 1 h in the same fixative to which 30% (w/v) sucrose was added. Afterwards, they were transferred to 30% (w/v) sucrose in PB for 12 h at 4 °C. For the DA-immunoreactivity study the same protocol was repeated with another three pigeons but using 1% of paraformaldehyde as well as 5% of glutaraldehyde as fixative. The dissected spinal cord was stored for 1 h in the same paraformaldehyde–glutaraldehyde fixative with 30% (w/v) sucrose. Afterwards, it was treated in the same way as described before for TH.

Spinal cords were cut in transversal or sagittal slices of 30 μm and collected in PB containing 0.05% (w/v) NaN_3 as a preservative. Transversal sections were made at levels C2, C7, C13, T1, T4, L1–L2, S1–S2, and S6–S7. Those for sagittal cuts were performed at C6, C14 and S3–S5. Treatment of animals conformed to the specifications of the German law for the prevention of cruelty to animals.

2.2. Immunocytochemistry

For the ABC (avidin–biotin complex) technique, slices were treated according to the following procedure:

Free floating sections were incubated overnight at 4 °C in anti-TH antibody (anti-TH) from mouse (Boehringer; working dilution 1:200) in PBS containing 0.3% (v/v) TritonX-100 (Sigma) pH 7.4; or anti-DA from rabbit (kindly provided by R.M. Buijs; Netherlands Institute for Brain Research, Amsterdam; working dilution 1:500) in Tris (0.05 M Tris buffer, 0.9% NaCl, 1% $Na_2S_2O_5$) containing 0.3% (v/v) TritonX-100, pH 7.2. The following steps were carried out at room temperature, separated by three washes of 2 min and two washes of 10 min in PBS (anti-TH) or Tris (0.05 M Tris, 0.9% NaCl; anti-DA). Slices were pre-incubated in 10% (w/v) rabbit (for TH) or goat (for DA) serum in PBS or Tris, respectively. After washing, slices were incubated for 1 h in the secondary antibody directed against mouse or rabbit IgGs (from rabbit or goat) diluted 1:200 (for anti-TH and anti-DA) in PBS containing 0.3% TritonX-100. After washing, slices were put for 1 h in Vectastain ABC-solution (Vector) in the same buffer as the former incubations. For TH, the normal washes were followed by an additional wash in 0.12 M acetate buffer (pH 6). Staining was achieved by the 3,3′-diaminobenzidine (DAB) technique with heavy metal (only for TH) amplification by adding $H_8N_2NiO_8S_2$ (2.5 g/100 ml), NH_4Cl and $CoCl_2$ (both 40 mg/100 ml).

For labeling with DAB, 400 mg/100 ml β-D-glucose were added to the solution. After 10 min of pre-incubation the reaction was catalyzed with 100–200 U/mg glucose-oxidase (Sigma, type VII). Due to the very fast reaction catalyzed, in some cases few drops of a 0.3% H_2O_2 solution were substituted for glucose/glucose-oxidase. Finally, sections were rinsed, mounted on gelatin-coated slides and coverslipped with Permount (Fisher Scientific, St. Louis, MO). Photographic documentation was carried out with an AxioCam color camera, handled with AxioVision 3.0 software (Carl Zeiss Vision, Hallbergmoos, Germany) attached to an Olympus BH2 or Leica DMRE microscope using DIC optics. Digital images were processed with PhotoShop 5.5 software (Adobe, Mountain View, CA). Contrast and brightness were adjusted with variable extent to meet satisfying output results with the Fuji 'MediaLab' printer device.

3. Results

3.1. TH-immunoreactivity

Two TH-positive cell populations were found at every transversal and sagittal level examined along the pigeons' spinal cord. Fig. 1a shows a schematic representation of the distribution of these TH-immunoreactive cells.

The first population were the LCNs, that were located in lamina VIII (according to Leonard and Cohen, 1975)

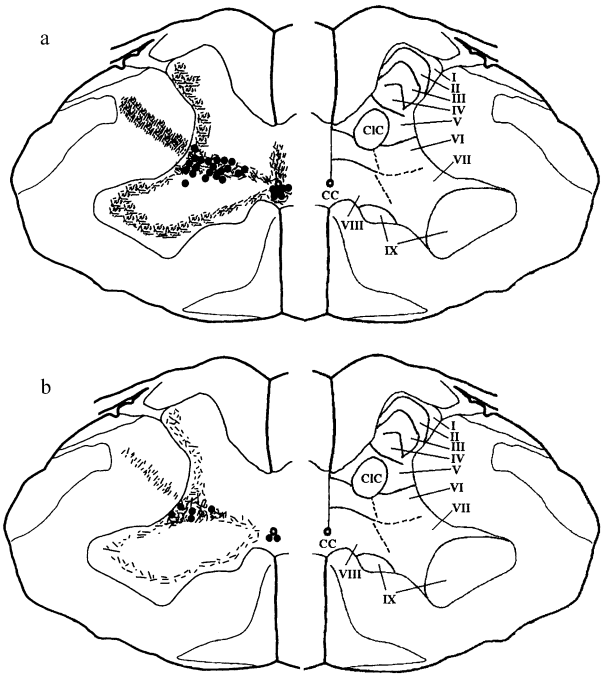

Fig. 1. Schematic diagram of a cross-section of the pigeon's spinal cord at the cervical level (C12). Dots symbolize body cells and lines symbolize fibers. (a) Distribution of TH-immunopositive neurons and fibers; (b) distribution of DA-immunopositive neurons and fibers. Layers and their denominations are according to Leonard and Cohen (1975). CLC is the Clarke's column and CC is the central canal. DA- and TH-immunopositive cell number corresponds to 25 slices across the spinal cord. Only a fraction of LCNs observed in these 25 sections could be shown. On average, LCNs were much more numerous than neurons of the dorsolateral column.

ventral to the lumen of the central canal (Fig. 2a and b). In sagittal sections they formed a continuous band along most levels of the spinal cord (Fig. 2c). The shape of these bipolar LCNs was ovoid with some of them having a process protruding into the central canal. Thus, they closely resembled the LCNs described by Roberts et al. (1995) for spinal cells of non-mammalian vertebrates. Looking at the longitudinal distribution, LCNs were more abundant in the rostral cervical (C2–C3) and sacral (S5–S6) regions, intermediate in the thoracic and the lumbar cord, and very scarce in the medial cervical region (C6–C7). At sacral levels, also a few displaced LCNs dorsal to the central canal were found. The second population of TH cells was located mainly in laminae VI and VII, dorsolaterally to the central canal (Fig. 3a and b). These multipolar cells were less numerous than the LCNs and slightly more scattered with respect to their location.

The TH fiber distribution is shown in Fig. 1a. TH fibers in the gray matter were present in a reticular fashion with higher densities around and above the central canal in lamina VIII, in the ventral horn close to the border to the white matter, in laminae VII–IX, and in the lateral part of the dorsal horn in laminae II–VI.

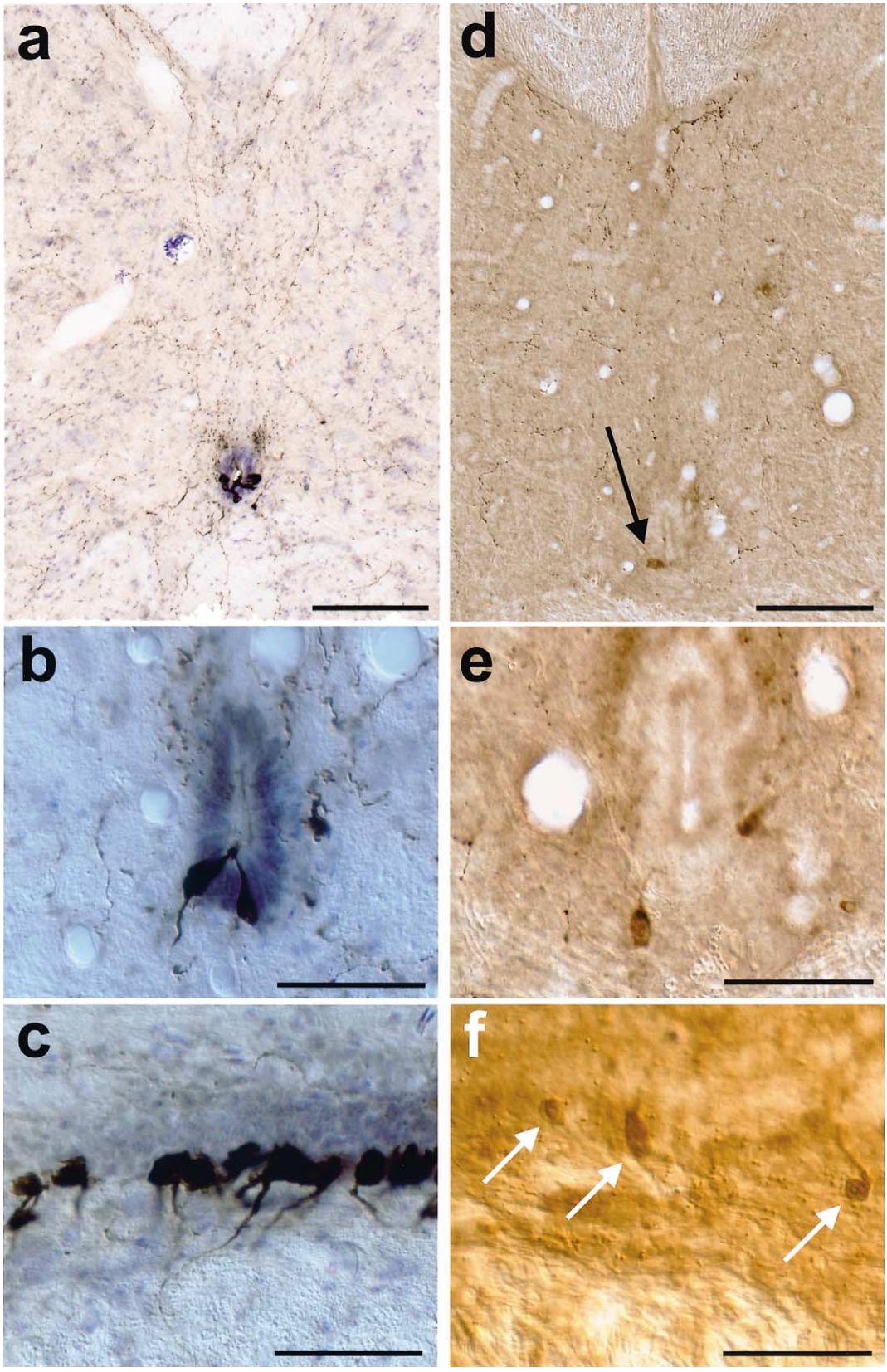

Fig. 2

TH fibers in the white matter were clearly longitudinally oriented and could be observed as a strong fiber tract at every level of the spinal cord in the dorsal part of the lateral columns.

3.2. DA-immunoreactivity

As schematically shown in Fig. 1b, two main cell populations of DA-positive neurons were found along the whole rostro-caudal axis of the spinal cord. Generally, DA-like cells were found at the same locations as TH-immunoreactive ones but were less numerous and not so homogeneously distributed.

The first group of DA-positive neurons was found in lamina VIII, contacting the central canal (Fig. 2d and e). The somata of these cells were located near the central canal with processes protruding into it, as also described for the European eel and the rainbow trout by Roberts et al. (1995). In sagittal slices these cells were visible as a row of about equidistant neurons ventral to the central canal and contacting the canal lumen (Fig. 2f). This was identical to the comparable TH picture with the sole difference that the dopaminergic cells were more sparse and thus more distant from each other. The DA-positive LCNs also showed a segmental distribution similar to the TH-positive cells. They were more abundant at rostral cervical and sacral levels, intermediate at thoracic and lumbar levels and rare at caudal cervical level. The second group of neurons was also present dorsolaterally to the central canal, coincident with the TH cells in lamina VI and VII (Fig. 3c–e). Like the TH-immunoreactive cells the DA-immunoreactive cells showed a multipolar shape. These lateral DA-positive cells were also more scarce than the DA-positive LCNs.

Fig. 1b shows the distribution of DA-immunoreactive fibers in the dorsal and the ventral horn. These fibers generally had an overlapping distribution with that of the TH-immunoreactive fibers. The only exception was that DA fibers were absent around the central canal in lamina VI where they could be observed with TH. It is also worth mentioning that the number of fibers labeled using anti-DA was lower compared using TH antibodies.

4. Discussion

The present immunocytochemical study shows that dopaminergic cells are present in the spinal cord of adult pigeons. In transverse sections, both TH- as well as DA-positive cells were located within two pools: One was situated at the borderline between dorsal and ventral horn (layers VI, VII), the second was located ventral to the central canal. Catecholaminergic cells clustered in the rostral cervical and sacral regions, with only few cells labeled at thoracic or lumbar levels.

The amount of DA-immunoreactive cells and fibers was lower than the amount of TH-immunoreactive cells and fibers, respectively. This may be due to two possible reasons. First, it is possible that only a fraction of catecholaminergic cells are dopaminergic. Indeed, some studies demonstrated noradrenaline to be about ten times more abundant in the spinal cord of rats than DA (Commissiong and Neff, 1979; Commissiong et al., 1978). Second, the sensitivity of TH detection could be higher due to technical reasons. To test our efficiency to label DA, we carried out the same protocol with more rostral brain preparations. These sections showed a very clear and strong labeling of DA cells in the substantia nigra and the ventral tegmental area. A profuse distribution of DA fibers was also found in the whole striatum and DA axons could be followed up to finest details in other forebrain regions. Since our technique was perfectly able to label DA processes, we suggest that only few TH-positive catecholaminergic cells and fibers within the spinal cord are of dopaminergic nature. This would correspond to the situation in other species.

4.1. Comparison of TH and DA distribution within vertebrates

The distribution pattern of TH-immunoreactive cells partially agrees with previous data from chicken. Wallace et al. (1987) found in embryonic and hatching chicken two conspicuous TH-immunoreactive cell populations: cells situated ventral to the central canal and cells mainly situated in the lateral border of the dorsal horn in the layers I, II, V and VI (according to Martin, 1979). Only cells situated in layer VI of chicks correspond to the TH-immunoreactive cells of pigeons situated in layers V and VI. However, in the dorsal horn of the pigeon no TH-immunoreactive cells, were found.

Wallace et al. (1996) found only very few DA-positive spinal cells in hatching chicks. These neurons were exclusively situated ventral to the central canal. In the pigeon spinal cord we found DA-positive neurons in the same location but also in layers V and VI of the

Fig. 2. Photomicrographs illustrating TH- and DA-immunopositive LCNs in the spinal cord of pigeons. (a) and (b) Transversal slices illustrating TH-immunopositive neurons located ventrally to the central canal in lamina VIII; (c) Sagittal slice illustrating a band of TH-immunopositive LCNs ventral to the central canal; (d, e) transversal slices showing DA-immunopositive LCNs located similarly to those of TH-immunopositive ones; (f) sagittal slice illustrating a row of DA-immunopositive LCNs ventral to the central canal and along the medial axis of the spinal cord. Mostly, these cells are roughly equidistant. In this picture, one neuron is outside the plane of focus. Arrows point to somata. Scale bar = 100 μm for a and d, 50 μm for b, c, e, and f.

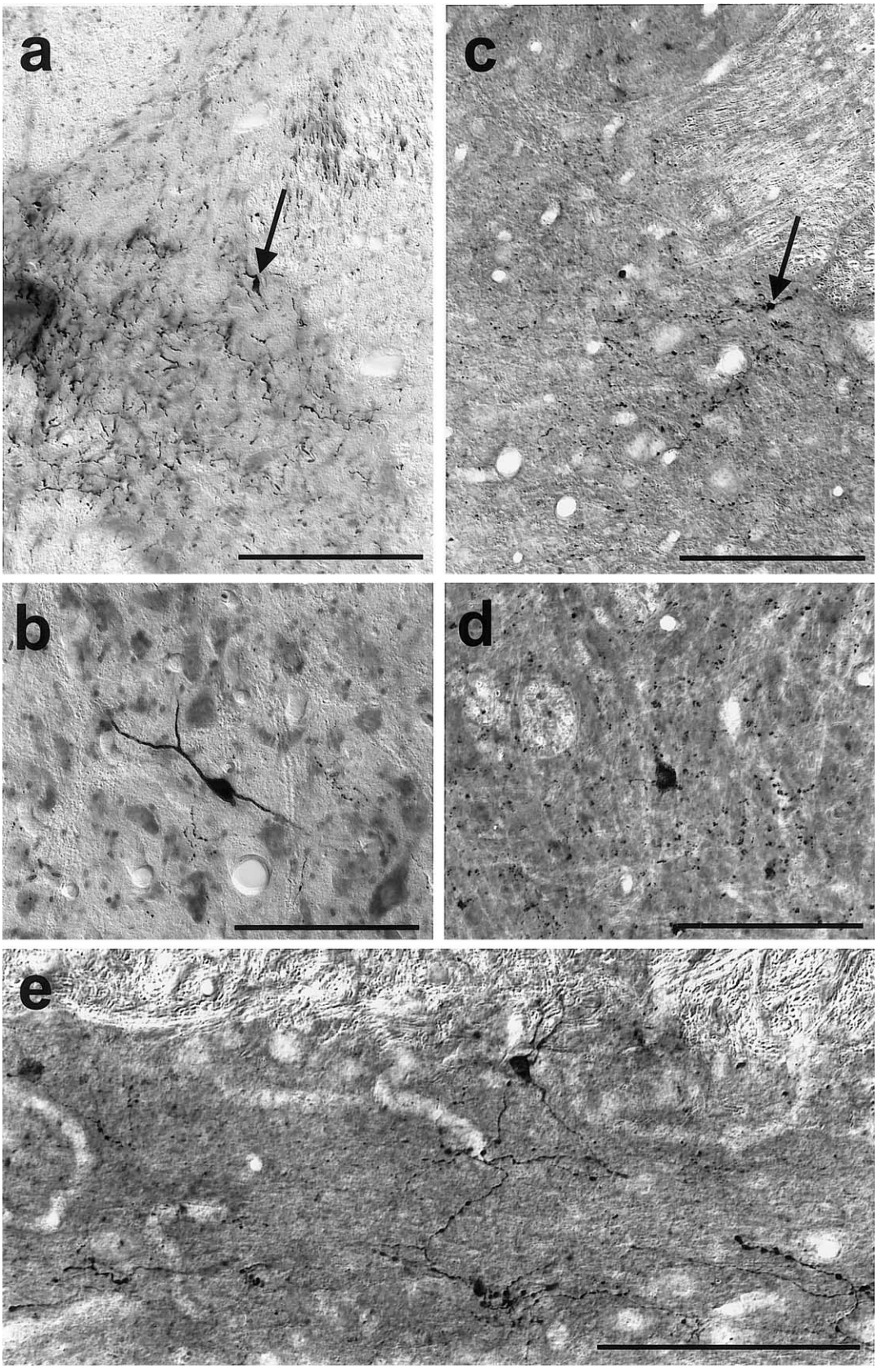

Fig. 3

mediolateral part of the gray matter. Experiments in chicks and pigeons differed concerning two major aspects: Besides the species difference, we investigated adult instead of newborn animals. In vertebrates the differentiation and the maturation of the catecholaminergic system is a relatively late ontogenetic event (Pindzola et al., 1990; Gonzalez et al., 1994). TH-immunoreactivity occurs before DA expression and the first brain structure that exhibits DA-immunoreactivity is the cell group ventral to the central canal (Gonzalez et al., 1994). In this respect, the differential DA-immunoreactivity observed between hatching chicks and adult pigeons might reflect a developmental gradient rather than interspecies pattern variation. This view is supported by the overall poor DA content in juvenile chicks, as DA-immunoreactive cells could only be detected after intracellular amplification via L-DOPA and MAO inhibitor treatment (Wallace et al., 1996).

The two TH- as well as DA-expressing spinal cell groups observed in the present study are also present in other vertebrate genera: The TH-immunoreactive LCNs were described in all vertebrates except mammals (Pierre et al., 1997; Wallace et al., 1996; Gonzalez et al., 1995, 1994; Guglielmone, 1995; Roberts et al., 1995; Okado et al., 1991; Stuesse et al., 1991; Smeets and Gonzalez, 1990; Chikazawa et al., 1983). These cells lie predominantly ventral to the central canal. They are bipolar in shape with a process protruding into the central canal and another process directed ventrally (Smeets and Gonzalez, 2000). These TH-expressing LCNs seem to constitute a primitive feature in vertebrate evolution and their absence in mammals (Uda et al., 1987; Smeets and Gonzalez, 2000) seems to be a derived feature.

By contrast, the existence and location of TH-immunoreactive cells that do no contact the central canal vary widely within different vertebrate classes. While lampreys exhibit a sub-population of lateral spinal cells (Pombal et al., 1997; Schotland et al., 1996), similar in location as described for birds (Okado et al., 1991, present study), cartilaginous fish lack such neurons (Stuesse et al., 1991; Roberts and Meredith, 1987) while they were described in teleosts (Roberts et al., 1989). Within the three amphibian orders, only anurans possess non-liquor contacting catecholaminergic spinal neurons (Sanchez-Camacho et al., 2001). Reptiles lack TH-immunoreactive cells beyond the LCNs (Kiehn et al., 1992; Bennis et al., 1990–91; Smeets and Steinbusch, 1990; Wolters et al., 1984) while the investigated mammalian species exhibit lateral spinal TH-immunoreactive cells with variable locations (Uda et al., 1987; Mouchet et al., 1986). Taken together, the lateral spinal TH-positive cell groups, present within different vertebrate classes, underwent several independent variations throughout vertebrate evolution.

There is no direct evidence for dopaminergic neurons in the spinal cord of mammals (Holstege et al., 1996; van Dijken et al., 1996; Mouchet et al., 1992). However, DA-positive cells in the spinal cord of non-mammalian vertebrates were observed repeatedly. In the lamprey they lie ventral as well as ventrolateral to the central canal (non-LCNs; Pombal et al., 1997; Pierre et al., 1997). In amphibians, DA-positive LCNs were found ventrally to the central canal (Gonzalez et al., 1994). In the European eel multipolar dopaminergic neurons were found in the dorsomedial portion of the spinal cord and DA-positive bipolar cells were found ventral to the central canal (LCNs; Roberts et al., 1989). In pigeons the DA-positive cells were found in similar locations as in lampreys and European eels. So, while mammals completely lack dopaminergic cells within the spinal cord, in all non-mammalian species studied so far, at least a sub-population of the catecholaminergic LCNs is of dopaminergic nature. This homogeneous picture does not apply for the lateral catecholaminergic cell group, where DA-immunoreactive cells were shown beneath the pigeon only within lampreys and one teleost.

4.2. Functional considerations

The TH and DA innervation of the spinal cord is partly of different origin in vertebrates. Whereas spinal DA fibers in mammals stem from supraspinal sources, in non-mammalian vertebrates parts of the DA innervation are of intraspinal nature. The function of spinal DA cells is far from understood, but, as outlined below, different roles in nociception, autonomic function, and motor control are conceivable. In the following, we will concentrate mainly on dopaminergic mechanisms.

4.2.1. Nociception

The presence of DA fibers and terminals in the dorsal horn might suggest the involvement of spinal DA in the processing of nociceptive information. Studies on nociceptive spinal reflexes in decapitated cats showed that the application of L-DOPA depressed monosynaptic reflexes of flexors muscles increasing the delay in the reaction to noxious stimulation (Schomburg and Steffens, 1998). In rats and cats, it was shown that the A11 dopaminergic cells that project to the spinal cord selectively suppressed nociceptive responses of spinal multireceptive neurons (Fleetwood-Walker et al., 1988). Since these studies were only performed in mammals

Fig. 3. Photomicrographs illustrating the lateral group of TH- and DA-immunopositive neurons in the spinal cord of pigeons. (a, b) transversal slices illustrating TH-immunopositive neurons in the lateral gray matter between layers VI–VII; (c, d) transversal slices illustrating DA-immunopositive neurons located identically to those of TH-immunopositive ones; (e) sagittal slice showing a DA-immunopositive lateral neuron in the gray matter. Arrows point to somata. Scale bar = 50 μm for a and c, and 100 μm for b, d, and e.

where an intraspinal DA system is lacking, it is not possible to speculate if the spinal DA system in pigeons also participates in nociceptive signalling.

4.2.2. Autonomic functions

Gladwell and Coote (1999) showed that the descending pathway from the hypothalamus (A11 cells in the rat) to the spinal cord has its main dopaminergic projection onto the sympathetic inter-mediolateral cell column and sympathetic pre-ganglionic neurons. After depleting spinal dopamine by chronic midthoracic transection in rats, Lahlou (2000) demonstrated that apomorphine, a DA agonist, enhanced hypotension and bradycardia when injected caudally but not rostrally to spinal transection. The author suggested a dopaminergic and spinal origin of the modulation of bradycardia and hypotension.

4.2.3. Motor control

The DA motor control in the spinal cord of vertebrates was studied in many species. In mammals, the descending projections from the diencephalic A11 cells to zones around the pre-ganglionic sympathetic neurons at the levels of the thoracic-lumbar nerves seem to modulate different motor reflex systems (Maisky and Doroshenko, 1991; Lindvall et al., 1983). Consequently, the DA agonist apomorphine and the DA metabolite L-DOPA mediate the flexor and the mass reflexes in rats and cats (Geber and Dupelj, 1977; Nygren and Olson, 1976). In rats, strong labeling of DA fibers occurs at the parasympathetic area and around two motor nuclei of the lumbrosacral cord (van Dijken et al., 1996). These motor nuclei are the spinal nucleus of the bulbus cavernosus, which innervates the external anal sphincter and the bulbocavernosus as well as the dorsolateral nucleus which innervate the external urethral sphincter (van Dijken et al., 1996; Smeets and Gonzalez, 2000). In lampreys low doses of DA injected into the spinal cord cause a rate acceleration of fictive swimming first induced by glutamate (McPherson and Kemnitz, 1994). All these examples might indicate a role of DA in the enhancement of the activity levels of motoneurons which were previously activated by other afferents. Indeed, Smith et al. (1995) showed DA in birds to be able to double glutamate-activated currents. This effect was contingent upon an activation of D1-receptors. According to Durstewitz et al. (1999) and Seamans et al. (2001) DA acts in prefrontal cortex via D1-receptors to enhance sustained synaptic inputs by increasing the NMDA component of EPSCs and by enhancing the persistent $Na+$ and reducing the slowly inactivating $K+$ currents of active neurons. The net effect of these modulations is a selective enhancement of the firing frequency of preactivated cells. Although a functional interpretation of the spinal DA pattern in birds is highly speculative at the present state of knowledge, it seems to be possible that the spinal DA systems in birds contribute to a stabilization and enhancement of the activity levels of motoneurons during locomotion and flight.

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