Histochemical localization of NADPH-diaphorase in the rat accessory olfactory bulb

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Abstract. The distribution of NADPH-diaphorase activity was examined in the accessory olfactory bulb of the rat using a direct histochemical technique. Labeled fibers and somata were found in all layers of the accessory olfactory bulb. The entire vomeronasal nerve and all vomeronasal glomeruli were strongly labeled, contrary to the main olfactory bulb, where only dorsomedial olfactory glomeruli displayed NADPH-diaphorase activity. NADPH-diaphorase positive neurons were identified as periglomerular cells in the glomerular layer and external plexiform layer, horizontal cells in the internal plexiform layer, and granule cells and deep short-axon cells in the granule cell layer. The labeled dendrites of the granule cells formed a dense neuropile in the granule cell layer, internal plexiform layer and external plexiform layer. The staining pattern in the accessory olfactory bulb was more complex than what has been previously reported, and demonstrated both similarities and differences with the distribution of NADPH-diaphorase in the main olfactory bulb.

Introduction

The accessory olfactory bulb (AOB) of the rat is a layered structure located in the olfactory peduncle, dorsal and slightly medial to the main olfactory bulb (MOB). Although both structures are easily comparable, the MOB and the AOB differ in their structure, connections and functional organization. Among the main morphological differences, the AOB shows a less organized laminar structure; its glomeruli are smaller and less distinct than the almost single row arrangement present in the MOB; the output neurons are more polymorphic than the MOB mitral cells and they do not appear in a monocellular layer as in the MOB, and the number of periglomerular cells and granule cells is reduced (Ramón y Cajal, 1902; Allison, 1953; Switzer et al., 1985).

The MOB and the AOB receive peripheral inputs from different groups of receptor neurons and project to different brain areas (White, 1965; Heimer, 1968; Price, 1973; Scalia and Winans, 1975, 1976; Kosel et al., 1981). Attending to their physiological role, whereas the main olfactory system is a selective recognition system for very heterogeneous kinds of odors, the accessory olfactory formation has been related to the recognition of some specific non-volatile substances such as pheromones (Wysocki et al., 1980; Sánchez-Criado et al., 1989). This later chemoreception mechanism between conspecifics is involved in newborn—mother recognition in early postnatal period (Teicher et al., 1984), and in social behaviors in adults, like mating, aggression and maternal behavior (Wysocki, 1979; Gallego et al., 1981; Bean and Wysocki, 1989).

NADPH diaphorase (ND) is a selective histochemical marker for specific neuronal populations throughout the vertebrate brain (Kowall et al., 1985; Sandell, 1985; Sagar, 1986; Sandell et al., 1986; Ellison et al., 1987; Cobcroft et al., 1989; Sato, 1990a,b; Arévalo et al., 1993). Although it has been reported that ND is the neuronal nitric oxide synthase (NOS) (Hope et al., 1991), and the localization of NOS immunoreactive
neurons in the central nervous system is absolutely coincident with ND staining (Bredt et al., 1991a), a recent study (Kishimoto et al., 1993) using ND histochemistry, NOS immunocytochemistry and in situ hybridization with NOS antisense oligonucleotide probes indicates that NOS is not expressed by either olfactory or vomeronasal receptor neurons. A role for nitric oxide in the receptor neurons has been proposed, both in the cilia and in the terminals (Breer and Shepherd, 1993; Lischka and Schild, 1993). The terminal fields of the olfactory and vomeronasal receptor neurons, the olfactory and vomeronasal glomeruli, have been reported as ND-positive (Davis, 1991; Vincent and Kimura, 1992; Alonso et al., 1993). It indicates that, at least in the olfactory and vomeronasal systems, ND activity labels NOS in intrinsic neuronal types and centrifugal fibers, and labels NADPH-P450 reductase in the sensory afferents (Kishimoto et al., 1993). There are three isoforms of NOS (see Schmidt et al., 1993, for a review) and cytochrome P450 reductase is the only known mammalian enzyme with a close homology with them (Bredt et al., 1991b). Cytochrome P450 reductase lacks the l-arginine binding site and it cannot, therefore, act in the arginine-to-nitric oxide pathway. Nevertheless, this latter enzyme may also be involved in the production of nitric oxide through a different biochemical pathway since cytochrome P450 catalyses the oxidation of Nω-hydroxy-l-arginine by NADPH and O2 to nitric oxide. Using this pathway, rat liver microsomes which exhibit no NOS activity are able to synthesize nitric oxide (Knowles et al., 1990).

Brain nitric oxide has been proposed as a retrograde messenger involved in synaptic plasticity events, like long-term potentiation (Böhme et al., 1991; Bon et al., 1992) and long-term depression (Shibuki and Okada, 1991). In the olfactory system, nitric oxide has been proposed as a mediator in the chemoelectrical transduction process in olfactory receptor neurons, in the recruitment of adjacent olfactory receptors, and in the intraglomerular synaptic integration of sensory inputs (Breer and Shepherd, 1993). In addition to olfactory axons, different types of interneurons, including periglomerular cells, superficial and deep short-axon cells and granule cells have been reported as ND positive (Scott et al., 1987; Villalba et al., 1989; Davis, 1991; Vincent and Kimura, 1992; Alonso et al., 1993) and NOS immunoreactive (Bredt et al., 1990; Kishimoto et al., 1993) in the MOB. One way to provide more evidence on the functional meaning of ND activity in the chemosensory systems is to establish whether the ND staining pattern is similar or different in the AOB and MOB.

Whereas the distribution of ND staining has been carefully detailed in the MOB (Scott et al., 1987; Bredt et al., 1991a; Davis, 1991, Vincent and Kimura, 1992; Kishimoto et al., 1993), and ND-positive neurons and fibers have been previously observed in the AOB (Scott et al., 1987; Davis, 1991; Vincent and Kimura, 1992; Kishimoto et al., 1993), no comprehensive study of their morphology and distribution is hitherto available. This paper provides a detailed description of ND-labeled elements in the different AOB layers, comparing them with previous reports on the same structure, and with the histochemical distribution of ND and the immunocytochemical distribution of NOS in the MOB.

Materials and methods

Five adult female Wistar rats weighing 220–250 g were used in this study. The animals were deeply anesthetized using ketamine (Ketolar, 50 mg/kg body weight), and perfused...
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intra-aortically with 100 ml heparinized saline solution followed by 400 ml of a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the skull and post-fixed for 4 h in the same fixative. Thereafter, the olfactory bulbs and olfactory peduncles were dissected out and stored overnight in phosphate buffer containing 30% (vol/vol) sucrose for cryoprotection. The tissue was frozen with liquid nitrogen and 30 μm coronal and sagittal sections were cut on a cryostat (Bright) at −21°C. The sections were rinsed in several changes of phosphate buffer and processed for the demonstration of ND as previously described (Alonso et al., 1992a,b, 1993). Briefly, the sections were incubated in a medium containing 1 mM β-NADPH (Sigma), 0.3 mM nitroblue tetrazolium (Sigma) and 0.1% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.0), at 37°C for 45–90 min. The course of the reaction was controlled under the microscope. Some sections were incubated for shorter periods (15–40 min) to visualize more clearly stained cells that were normally obscured by the dense neuropile staining observed in the glomerular layer and the granule cell layer. After incubation, the sections were rinsed in phosphate buffer, dehydrated in a graded ethanol series, cleared with xylene and mounted with Entellan (Merck). Controls for specificity of the ND reaction were carried out as previously described (Alonso et al., 1992a,b, 1993). No ND activity was observed when the tissue was incubated without NADPH or without the electron acceptor nitroblue tetrazolium. Cell sizes were measured using a Zeiss ocular micrometer.

Results

The AOB is a laminar structure where five layers can be differentiated. Axons of the vomeronasal receptor cells form the vomeronasal nerve layer (VL). In the glomerular layer (GL) these axons contact with dendrites of mitral cells and periglomerularly located cells. The mitral cells are dispersed in the external plexiform layer (EPL) contrary to what is observed in the MOB, where they form a single cell layer. The internal plexiform layer (IPL) is masked by the lateral olfactory tract, which contains the axons of output neurons located in the MOB and AOB. Numerous granule cells, sending their main dendrites to contact with mitral and periglomerular cells, are situated in the innermost layer, the granule cell layer (GCL).

The ND histochemical technique provides a Golgi-like labeling of cells and fibers, enabling a detailed morphological characterization of positive elements. In the AOB, ND activity was observed as numerous positive cell bodies and fibers located in the GCL, a few scattered neurons located in the GL, EPL and IPL and a neuropile staining in all layers, specially dense in the VL, GL and GCL. Figure 1 shows schematically the distribution of ND-positive elements in the AOB and MOB.

All vomeronasal axons were ND-active. They arrived at the dorsal surface of the AOB, and arborized in spherical or elliptical glomeruli. The staining intensity was heterogeneous between different glomeruli, but all of them were labeled (Figure 2a). These glomeruli were irregular in size (50–100 μm of main diameter) and shape, and they were arranged in two or three different rows.

In sections incubated for shorter periods, scarce ND positive neurons were observed among the glomeruli (Figure 2b–e). These cells were weakly labeled, with diameters ranging between 7 and 10 μm, and generally round, more rarely ovoid shapes. The negative nucleus occupied most of the cellular volume. These ND labeled cells were
normally located at the deep side of the glomeruli, i.e. close to the EPL. According to their location and morphological characteristics they were identified as periglomerular cells. These positive periglomerular cells were morphologically heterogeneous, including monodendritic and bidendritic cells. In some cases, these cells had one long stained dendrite that could be followed into one glomerulus where it branched profusely.

In the EPL, very few ND-active periglomerular displaced neurons were observed (Figure 2f). These cells had small, round cell bodies and one long dendrite directed towards the GL, where it branched. Neither mitral cells nor superficial short-axon cells were observed after ND-staining. ND-labeled superficial short-axon cells were clearly observed in the adjacent MOB. In the IPL, small (7—10 μm of mean diameter) ND-positive cells were found, demonstrating two different morphologies: horizontal cells with oval somata orientated along the negative fibers of the lateral olfactory tract (Figure 3a) and spherical cells with a single dendrite transversely oriented to the axons.
Fig. 2. (a) Panoramic view of a coronal section of the rat AOB after ND histochemical staining. Note the presence of ND activity in all vomeronasal fibers and glomeruli, as well as the intense neuropile staining in the granule cell layer. VL—vomeronasal nerve layer. GL—glomerular layer. EPL—external plexiform layer. IPL—internal plexiform layer. GCL—granule cell layer. Scale bar = 100 μm.
(b–f) ND activity in the superficial layers of the rat AOB. Scale bar = 50 μm. (b) ND-active vomeronasal fibers and glomerulus (G). Scattered labeled fibers can be observed in the external plexiform layer (EPL). (c) ND-positive periglomerular neurons surrounding a glomerulus with dendrites branching close to the cell body. (d) Positive periglomerular cell with a long dendritic trunk. (e) ND-active bidendritic periglomerular cell. (f) Displaced periglomerular neurons (arrows) located in the external plexiform layer.
Fig. 3. ND-active neurons in the deep layers of the AOB. Scale bar for all figures = 50 μm.
(a) Sagittal view of the internal plexiform layer of the AOB, where a ND-positive horizontal cell (arrow) was observed. (b) Magnification of the boundary of the granule cell layer and the internal plexiform layer. Intensely stained dendrites of granule cells and a granule cell soma (arrow) can be observed. (c) ND staining in the granule cell layer, showing positive somata and dendrites (arrows) of granule cells. Labeled terminal puncta can be observed. (d) Positive granule cell at the rostralmost extent of the AOB. The spiny main dendrite can be clearly observed. (e) ND-active deep short-axon cell located in the AOB granule cell layer. (f) ND-positive neuron located at the deepest portion of the granule cell layer, with a long dendrite reaching the anterior olfactory nucleus (arrow).
of the lateral olfactory tract, coursing towards the EPL (Figure 3b). These latter ND-active neurons were identified as displaced granule cells.

Two different neuronal populations of ND-labeled cells were differentiated in the GCL, according to their size, morphology and staining intensity. The first group was formed by very abundant neurons with weakly stained somata distributed throughout the whole GCL. These neurons were spherical, small in size (7–10 μm of diameter), and with a clearly observable ND-negative nucleus. A single and spiny dendrite could be observed arising from the dorsal pole of the cell body. Axon-like prolongations were not observed. According to their number, morphological characteristics and location, these neurons were identified as granule cells. All or most granule cells seemed to be ND-positive (Figure 3c), in contrast with the MOB where only a subpopulation of granule cells was ND-labeled. Their main dendritic processes could be distinguished crossing the IPL and reaching the EPL as dense bundles of ND-positive fibers (Figure 3a,b). In the EPL, the ND-positive dendrites surrounded negative profiles of mitral cells (Figure 2a). At rostral levels of the AOB, where only the GCL could be observed, individual granule cells with a long and spiny main dendritic trunk may be distinguished (Figure 3d).

In addition to granule cells, a population of strongly stained neurons appeared in the GCL. These neurons were mainly located in the deeper portions of the GCL. Some of them could be observed at the boundary of this layer and the adjacent anterior olfactory nucleus. These cells had elliptic somata ranging between 20 and 25 μm in diameter, and possessed long and varicose dendritic trees. Because of the dense ND reaction product located in the cytoplasm, the cell nucleus could not be observed. In some occasions, axon-like processes could be seen emerging from the soma or the initial portions of the dendritic trunk. These ND-active neurons were identified as deep short-axon cells (Figure 3e). These cells were much less abundant (about 1 or 2 per section) than the ND-stained granule cells. Their location, orientation and branching pattern resembled that of Blanes and Golgi types described in the MOB using Golgi impregnation. The long dendrites branched in a large extent of the GCL, and in some occasions they reached the dorsal subdivision of the anterior olfactory nucleus (Figure 3f). Finally, a uniform staining of fibers and terminal puncta was found in the GCL (Figure 3c). However, the fibers of the lateral olfactory tract crossing the IPL were always ND-negative (Figure 3a,b).

Discussion

This paper provides a description of ND-positive neurons and fibers in the rat AOB. Studies on the general distribution of ND activity throughout the brain (Vincent and Kimura, 1992), and reports mainly focused on the distribution of this enzyme in the MOB (Scott et al., 1987; Davis, 1991; Kishimoto et al., 1993), mentioned the presence of ND staining in the AOB. Our results show both similarities and differences with the ND distribution pattern described by these authors. Thus, granule cells and deep short-axon cells have been described as ND-active neurons in the AOB (Scott et al., 1987; Davis, 1991; Vincent and Kimura, 1992; Kishimoto et al., 1993) in agreement with our observations. Other neuronal types have been previously referred as being ND-negative, whereas we have observed for the first time scarce ND-positive horizontal cells and a subpopulation of periglomerular cells showing ND-positive labeling. One
possible explanation is that the dense fibrillar staining normally masked weakly-labeled ND-positive horizontal cells and periglomerular cells. Therefore, the positive staining of a subpopulation of periglomerular cells is a common characteristic between the MOB and the AOB, instead of a differential staining as previously considered. Previous data on the ND-labeling of vomeronasal fibers and glomeruli are contradictory: Scott et al. (1987), Davis (1991) and Kishimoto et al. (1993) reported positive staining of all of them, as observed in our sections, whereas Vincent and Kimura (1992) did not find ND activity in the VN and GL of the AOB. The discrepancy could be due to the short incubation period used by these latter authors, about 30–60 min, whereas the other groups and ourselves employed periods over 60 min. In this sense, Davis (1991) indicated that the reaction product varies from light to dark blue in the same neuron depending on the duration of the incubation. We have observed a temporal sequence in the appearance of positive elements in the AOB. After 30 min of incubation, ND-positive staining was observed in granule cells and deep short-axon cells. Then, periglomerular cells were detected, and after 80 min all vomeronasal fibers and glomeruli demonstrated ND activity. Longer periods only provide an increase in the unspecific background staining.

Comparing our results in the AOB with the distribution of ND activity in the MOB (Scott et al., 1987; Croul-Ottman and Brunjes, 1988; Villalba et al., 1989; Davis, 1991; Vincent and Kimura, 1992; Alonso et al., 1993; Kishimoto et al., 1993), differences and similarities in the staining patterns in these two structures can be observed. In both bulbs, periglomerular cells, horizontal cells and deep short-axon cells were ND-active. However, there are no ND-labeled superficial short-axon cells in the AOB. Furthermore, important quantitative differences in the number of ND-positive elements were noted. Thus, ND-active periglomerular cells were more abundant in the MOB than in the AOB, whereas the number of positive granule cells is clearly higher in the AOB than in the MOB. In addition, all vomeronasal fibers and glomeruli were heavily labeled, but only a subset of dorsomedial olfactory fibers and glomeruli was selectively stained (Croul-Ottman and Brunjes, 1988; Villalba et al., 1989; Davis, 1991; Alonso et al., 1993; Kishimoto et al., 1993).

In a recent work on the distribution of NOS in the mouse MOB and AOB Kishimoto et al. (1993) found a discrepancy between the ND and NOS stainings in the MOB glomeruli. Whereas the ND and NOS labelings were coincident in all intrinsic neuronal types, no NOS signal could be detected in the olfactory fibers and glomeruli. They attributed ND activity in the olfactory fibers to another enzyme, the NADPH-P450 oxidoreductase, suggesting that the topographical distribution of ND-positive and ND-negative glomeruli reflects specific odor stimulation and neuronal activity, similar to that detected with the 2-deoxyglucose method (Jourdan et al., 1980). However, the pattern of ND labeling in the olfactory glomeruli seems to be stable and stained glomeruli are always observed in the dorsomedial surface of the rat MOB (Croul-Ottman and Brunjes, 1988; Villalba et al., 1989; Davis, 1991; Alonso et al., 1993). Moreover, a similar pattern is observed in different species such as rat, hamster and mouse (Davis, 1991; Kishimoto et al., 1993). Even in odor deprivation experiments in rat pups, the distribution of ND-positive and ND-negative glomeruli was not altered at different ages and deprivation conditions (Croul-Ottman and Brunjes, 1988). In addition, all vomeronasal glomeruli demonstrated ND labeling, indicating according to that hypothesis.
that all of them would be simultaneously and continuously activated. These data suggest that the selective ND staining could be due to the activity of an enzyme only expressed in the positive glomeruli, but not to specific stimulation of a subpopulation of receptors.

Previous works on the immunocytochemical detection of different carbohydrate epitopes with monoclonal antibodies demonstrated a heterogeneous staining in olfactory and vomeronasal glomeruli (Allen and Akeson, 1985; Fujita et al., 1985; Imamura et al., 1985; Mori et al., 1985; Mori, 1987; Mori et al., 1987; Schwarting and Crandall, 1991). One of them, called CC2, shows a staining pattern in both MOB and AOB very similar to the distribution of ND activity since it is expressed in dorsomedial olfactory glomeruli and all vomeronasal glomeruli (Schwarting and Crandall, 1991). It has been proposed that this superficial carbohydrate antigen acts in the guidance of axons from the olfactory or vomeronasal neuroepithelium to their targets in the MOB and AOB (Schwarting and Crandall, 1991; Schwarting et al., 1992). In this case, CC2 antigen and/or nitric oxide could be involved in the maintenance of a spatial-specific connection from the neuroepithelium to the bulb during olfactory and vomeronasal receptor turnover.

It has been proposed that nitric oxide alters within the glomerulus of the MOB the neuronal response to odor molecules stimulation in the olfactory mucosa (Breer and Shepherd, 1993). Although only dorsomedial MOB glomeruli are ND-positive, all glomeruli are able to produce nitric oxide, due to the presence of ND-positive and NOS-immunoreactive periglomerular and short-axon cells surrounding them (Alonso et al., 1993; Kishimoto et al., 1993). The same general mechanism could be present in the AOB, but the modulatory possibilities of the model are clearly lower since all vomeronasal glomeruli were positive, no labeled superficial short-axon cells were observed and only a few periglomerular neurons demonstrated ND activity in the AOB. The absence of clear-cut regional heterogeneity could be due to the fact that the vomeronasal system is less involved in the discrimination between different odors compared with the MOB.

In the AOB, granule cells, the main modulatory intrinsic neurons, have been described as GABAergic (Mugnaini et al., 1984; Takami et al., 1992). These neurons probably colocalize GABA and NOS, since most or all AOB granule cells were ND-active, and displayed NOS immunoreactivity and NOS mRNA signal (Kishimoto et al., 1993). Thus, granule cells in the AOB may contain a dual system of interaction and modulation of mitral cells. One, based on the exocytic release of GABA on dendrodendritic synapses located in the EPL (Price and Powell, 1970). The second one, based on the release by diffusion across the membrane of nitric oxide, affecting different types of cells located in the proximity of ND-active neurons and processes. This dual system increases the possibilities of modulation in the bulb circuitry.

Concerning the afferent pathways, we have demonstrated the presence of ND-positive terminals in the GCL of the AOB. The AOB is reciprocally connected with all regions where it projects, including the bed nucleus of the accessory olfactory tract, the bed nucleus of the stria terminalis and the amygdala (Scalia and Winans, 1975, 1976; De Olmos et al., 1978). In addition, inputs from the horizontal and vertical limbs of the diagonal band of Broca have been previously described (Davis and Macrides, 1981). In this aspect, ND-active neurons have been detected in the medial amygdaloid nucleus, the bed nucleus of the stria terminalis (Vincent and Kimura, 1992), and in the vertical and horizontal limbs of the diagonal band of Broca (Davis, 1991; Kitchener and
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Diamond, 1993), suggesting these cells as possible sources of ND-positive terminals in the AOB.

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