Topographical Distribution of Reduced Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase in the Brain of the Japanese Quail


ABSTRACT

The distribution of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity was histochemically investigated in the Japanese quail brain. This enzyme is now considered responsible for the synthesis of nitric oxide, a novel neural messenger whose distribution has not been described in the avian brain until now. The histochemical technique provides a simple and reliable method for staining selected populations of neurons throughout the avian brain. In the telencephalon several regions showed heavily stained NADPH-diaphorase positive neurons and processes. In particular the paleostriatal-paraolfactory lobe complex showed the greatest presence of both positive cells and processes. Neurons and processes were also observed in several regions of the hyperstriatum as well as in the archistriatal nucleus taeniae. Some regions, such as the ectostriatum and the hippocampus, had no positive elements. In the diencephalon, the magnocellular hypothalamic system, which in mammals shows NADPH-diaphorase activity, did not show any particular accumulation of reaction product. On the contrary, retinorecipient areas, such as the visual suprachiasmatic nucleus and the lateral geniculate complex, displayed a composite structure of both positive neurons and processes. The brainstem revealed a large NADPH-diaphorase positive population extending through the tegmental nuclei to the locus coeruleus and subcoeruleus. A complex organization was also observed in the optic lobe, where fusiform elements were distributed within the stratum griseum and superficialis of the tectum. In the medulla, a dense terminal field was observed at the level of the nucleus of the solitary tract, whereas scattered neurons were located within the reticular nuclei. Although the staining of neurons and tracts was highly selective, the positive cells did not correspond to any single known neurotransmitter, neuropeptide, or neuroactive molecule system. Several sensory pathways were heavily stained for the NADPH-diaphorase, including part of the olfactory, visual, and auditory pathways. The findings of the present study reveal that the NADPH-diaphorase-containing systems in the avian brain are organized according to a pattern comparable, because of its complexity, to that observed in mammals. However, important interspecific differences suggest that this novel neural system might be involved in diverse tasks.

Key words: nitric oxide synthase, avian brain, Coturnix japonica, olfactory pathway, optic pathway

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NO is formed from the amino acid L-arginine in NADPH-dependent reaction catalyzed by NO synthase (NOS). NOS isoforms have been purified and characterized from the brain (Bredt and Snyder, 1990), macrophages (Stuehr et al., 1991), and endothelial cells (Pollock et al., 1991). Molecular cloning and the study of immunological properties have provided evidence that there are at least three types of NOS (Bredt and Snyder, 1990). Among these types, only neuronal NOS had been detected within the central and peripheral nervous system and had appeared colocalized with NADPH-diaphorase activity (Dawson et al., 1991).

Although partial colocalization of ND with different neurotransmitters and neuropeptides, such as neuropeptide Y (NPY) and C-flanking peptide of NPY, somatostatin, substance P, choline acetyltransferase (ChAT), tyrosine hydroxylase, enkephalins, GABA, vasopressin, oxytocin, and calbindin D-28k, has been described (Vincent et al., 1983a–c; Reiner and Vincent, 1987; Villalba et al., 1988; Schobert et al., 1989; Mizukawa et al., 1989; Caballero-Bleda et al., 1991; Alonso et al., 1992a,b; Sánchez et al., 1994), the overall distribution of ND does not fully match that of any substance so far described. On the other hand, ND histochemistry is an excellent neuroanatomical tool, since positive neurons appear stained in particular brain areas and nuclei in a “Golgi-like” way, demonstrating most of the entire dendritic tree, lengthy axons, and axonal terminals.

Most of the studies hitherto available on the distribution of ND have been carried out on mammals, mainly rodents (Scott et al., 1987; Villalba et al., 1988; Davis, 1991; Vincent and Kimura, 1992), carnivores (Reiner and Vincent, 1987; Mizukawa et al., 1989) and primates (Nakamura et al., 1988; Mufson et al., 1990). Important interspecies differences have been observed, even in closely related species, such as rat and hamster (Davis, 1991). However, little attention has been paid to the nonmammalian brain. So far there have been few studies on the avian central nervous system.
system and these have been limited to certain regions (Sato, 1990; Montagnese et al., 1991).

On the basis of the above information, in the present study we report on the histochemical distribution and morphological characterization of the ND-positive neurons in the brain of Japanese quail, a frequently investigated avian species.

**MATERIALS AND METHODS**

**Tissue preparation**

Four adult (two males, two females) Japanese quail (Coturnix japonica) were used in this study. Birds were bought at the age of 3 weeks from a local breeder (Oselli, Torino, Italy). At their arrival in the laboratory, the birds were isolated in individual cages, provided with food and water ad libitum, and exposed to 16 hours of light each day until sacrifice.

The animals were transcardially perfused with deep anaesthesia (Hypnodil; Janssens Pharmaceutica, Berse, Belgium; 50 mg/kg body weight) with 50–100 ml NaCl 0.9%.

Two quails (one male, one female) were perfused with 200 ml of a fixative composed of 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.2–7.4 (PB). The other two birds (one male, one female) were perfused with 200 ml of a fixative composed of 4% paraformaldehyde in PB.

Thereafter, the brains were dissected out, placed in the same fixative for 12 hours at 4°C, and immersed for 48 hours in a cryoprotective solution of 30% sucrose (w/v) in PB. Thirty-micrometer-thick sections were cut on a cryostat in the frontal plane and collected in cold (4°C) PB or directly on gelatin-coated slides.

**NADPH-diaphorase histochemistry**

Free-floating or mounted sections were incubated in a solution containing 1 mM reduced β-NADPH, 0.8 mM nitroblue tetrazolium and 0.08% Triton X-100 in 0.1 M Tris buffer (pH 8), at 37°C for 40–60 minutes. The reagents were obtained from Sigma, and the course of the reaction was followed under the microscope. Some sections were incubated without β-NADPH. After incubation, the sections were rinsed in PB, dehydrated in an ethanol series, cleared with xylene, and mounted with Entellan (Merck). Every third section was Nissl stained with toluidine blue in two anterior, fasciculus longitudinalis medialis, tractus septo-mesencephalicus, the majority of cranial nerve bundles).

**RESULTS**

Neuronal cell bodies and processes exhibiting NADPH-diaphorase (ND) staining were found in particular nuclei throughout the quail brain. Glial elements were not stained by this histochemical technique, whereas endothelial elements lining cerebral blood vessels were stained lightly. Control sections incubated without substrate did not show any staining of either neuronal elements or endothelial cells.

**General morphological characteristics**

In the Japanese quail brain, the ND-positive neurons exhibited different morphological features and intensity of the staining. Generally, the soma, dendrites, axons, and terminal fields were stained, whereas the nucleus of the cells was unstained. According to differences in the morphological characteristics and staining, we distinguished three types of neurons which can be classified as follows.

**Type I.** Several groups of medium-sized to large neurons were extremely heavily stained. They were generally stained in a Golgi-like manner, and the reaction was so strong that the nucleus of the cell was not clearly visible (Fig. 1A). Their processes were of various lengths and morphology, sometimes bearing small dendritic spines. These elements were mainly observed in the paleostriatum augmentatum (PA) and in other prosencephalic sites, in the nucleus tegmenti pedunculopontinus (TP), in the area ventralis of Tsai (AVT), or in the locus coeruleus (LC).

**Type II.** Several small to medium-sized neurons were less intensely stained, although still exhibiting a Golgi-like appearance, and were provided with a clearly unstained nucleus (Fig. 1B). They were located chiefly in the diencephalon, mesencephalon, and pons.

**Type III.** Many small to medium-sized neurons were only weakly stained. In this case the processes of the neurons were totally unstained or weakly stained (Fig. 1C). These cells were dispersed throughout the brain, sometimes mixed with the first two types, or clustered as in the dorsal thalamus, or the cerebellar granular layer.

Marked differences in staining intensity of ND-positive neurupole were also observed. They were mostly related to a different density of positive fibres or fibre endings (puncta) in the terminal fields. The most intensely stained terminal fields were observed in the nucleus habenularis lateralis (HL; Fig. 7C) and in the nucleus tractus solitarii (S; Fig. 11A), where the density of the reaction was high enough to prevent the discrimination of single fibres. The PA too showed a very high density of positive processes (Fig. 4A,B). Many other regions of the brain showed different degrees of histochemical staining, and only a few areas (i.e., the ectostriatum (E), the hippocampus (H), the nucleus accumbens, the nucleus rotundus (ROT), the nucleus mesencephalicus lateralis, pars dorsalis (MLd)) exhibited an almost total absence of fibres. The stained fibres were very few or absent within several large myelinated tracts (commissura anterior, fasciculus longitudinalis medialis, tractus septo-mesencephalicus, the majority of cranial nerve bundles).

**Distribution of NADPH-diaphorase-positive structures**

ND-positive structures (cell bodies and fibres) were distributed throughout the whole quail brain, even if most neurons were completely unstained with this technique. The morphology of neurons, their distribution, and that of terminal fields were not affected by the different fixatives, nor by individual variations, but were strikingly dependent on the topographical location. No obvious and distinct sexual difference was observed in the present, limited number of birds.

The distribution of ND-positive elements is reported in Figures 2 and 3, showing different levels of the quail brain. The nomenclature adopted in the present study is largely based on that developed by Kuenzel and Masson (1988) for the chicken brain, with some minor modifications (see Asté et al., 1991) concerning the identification of pontine catecholaminergic groups (Guggiulome and Panzica, 1982, 1984) and of the preoptic region (Panzica et al., 1991b).

**Telencephalon.** The telencephalon (Fig. 2A–H) contained a wide population of ND-positive cell bodies in the quail brain, with marked differences in the distribution and staining intensity of positive processes (axons and den-
The olfactory bulbs showed only a few, scattered positive fibres. Several intensely stained neurons (type I), medium-sized, with prominent short processes, associated with a neuropile showing high density of positive processes, were, however, observed in the ventral regions of the olfactory pathway (tuberculum olfactorii, lobus parolfactorius). At more caudal and dorsal levels, the staining intensity of the neuropil increased, reaching higher levels in the PA, which is totally outlined by the ND-positive elements (Fig. 4A). The staining intensity was due to the presence of a dense network of fine, varicose fibres, intermingled with few, thick, dendritic processes. The neurons were of the type I, large, generally multipolar, provided with long dendrites (Fig. 4B). The surface of dendrites was usually smooth, although in a few cases short dendritic spines were stained. The PA was the telencephalic location with the highest ND staining of the neuropile, and the largest number of positive neurons. Close to PA, the paleostriatum primitivum showed a large number of type I neurons like those of the PA, but the staining of the neuropile was at lower levels (Fig. 4C). Similarly, several type I neurons were observed in the whole extent of the paleostriatum ventrale (PVT; Fig. 6A,B).

The hyperstriatal areas had a dense network of fine varicose processes stained for ND. Large, multipolar, type I neurons were clustered along the medial aspect of the hyperstriatum accessorium (HA; Figs. 4L4, 5A,B). A few, weakly stained, small elements were also present. More caudally, the dense network of fibres extended to the hyperstriatum ventrale, and the more superficial layers of the neostriatum. Scattered, large, multipolar type I neurons were observed within the whole region. Their morphology resembled that of neurons in the HA, with very long, thin, and only moderately branched dendrites (Figs. 5C,D). In a few cases dendritic spines were observed. Several weakly stained, small neurons of type I1 were intermingled with the largest elements (Fig. 5D).

Neurons and fibres were more numerous and dense along the lines subdividing the avian telencephalic regions (lamina frontalis superior, lamina medullaris dorsalis, lamina hyperstriatica; Fig. 5G), and close to the lateral wall of the telencephalic ventricles (Fig. 5E). Caudally, the density of the network increased in the neostriatum caudale, whereas fibres were almost totally absent within the Hp.

The septal region showed several cell clusters formed by medium-sized to small, intensely stained type II elements, very tightly packed and embedded in an extremely dense positive neuropile. This situation made it impossible to describe the morphology of these small neurons in detail. The major groups were the nucleus septalis medialis (SM; Fig. 5F) and the nucleus commissurae pallii (nCPa; Fig. 5H). Loosely packed, larger multipolar type I and II neurons, provided with long dendrites bordering the lateral prosencephalic bundle, were observed within the caudal PVT (Fig. 6A).

**Fig. 2.** A–H: Eight cross-sectional drawings of the quail telencephalic, diencephalic, and mesencephalic areas arranged from the most rostral (A, telencephalon) to the most caudal (H, mesencephalon). Various morphological types of NADPH-diaphorase positive neurons and the density of stained processes of terminal fields are identified by different symbols and reported on the right side of the drawings (see legend to Fig. 3 for explanations of symbols). The location of nuclei identified in Nissl-counterstained sections is reported on the left side.
Figure 2
Fig. 3. A–H: Eight cross-sectional semischematic drawings of the quail mesencephalic, pontine, and medullaris areas arranged from the most rostral (A, mesencephalon) to the most caudal (D, medulla). Various morphological types of NADPH-diaphorase positive neurons and the density of stained processes of terminal fields are identified by different symbols, explained at the bottom of the figure, and reported on the right side of the drawings. The location of nuclei identified in Nissl-counterstained sections is reported on the left side.
Small type III neurons, sometimes showing short dendrites, were observed within the whole septal region, and in particular in the nucleus septalis lateralis, which was also filled with a low density network of positive punctate structures (Fig. 5F).

**Diencephalon.** ND-positive elements were also observed within the quail diencephalon, where they were limited to a few cell clusters, and a diffuse, low intensity network of varicose fibres (Fig. 2D–H). The positive diencephalic neurons were, with a few exceptions, small, bipolar or multipolar, with a variable degree of staining (type II and III elements).

Scattered, type II, medium-sized neurons were found in the anterior preoptic area. These elements were located close to the tractus septomesencephalicus, in a region partly covering the nucleus preopticus dorsalis, and partly the nucleus preopticus anterior. Weakly stained, smaller type III neurons were also present in this region. The neuropile showed low density of positive structures, represented by isolated varicose fibres and puncta. Many type III elements were observed within the nucleus preopticus medialis. A large cluster of medium-sized, heavily stained, multipolar type I and II neurons occurred laterally to it. This cluster merges dorsally with the cellular group occupying the caudal PVT (Figs. 6A, B). A group of small type II neurons was observed close to the top of the ventricle, and at the level of the commissura anterior. These neurons appeared to form a subdivision of the larger group occupying the nCPa (Figs. 5H, 6B). Only a few, scattered, small type III neurons were found periventricularly, with the exception of a cluster located within the nucleus anterior medialis hypothalami. In particular no stained cells were observed within the periventricular or lateral groups belonging to the vasotocin (VT)-mesotocin system (for major details of this peptidergic system, see Viglietti-Panzica, 1986; Sanchez et al., 1991). More caudally, intensely stained, small, type II elements were found in the lateral hypothalamus. Few, weakly stained, small neurons were observed within the nucleus decussationis supraopticae, pars ventralis (Fig. 6D), which is considered the avian homologue of the suprachiasmatic nucleus (m. suprachiasmaticus, pars lateralis, SCN1; Kuenzel and Masson, 1988). Many varicose, positive fibres were observed within the decussatio supraoptica, as well as within the SCN1 and more laterally within the nucleus geniculatus lateralis, pars ventralis (Fig. 6C). The larger hypothalamic groups were represented by two clusters located within the tuberoinfundibular region. These two groups showed an arc-like distribution, occupying portions of the regio lateralis hypothalami and nucleus ventromedialis (Fig. 6E). These two groups showed a different morphology. Medium-sized to large, bi- or multipolar, type I or II neurons were loosely arranged within the dorsal group (Fig. 6F). Small, weakly stained elements of type III occupied a more ventral position (Fig. 1C). Both groups were embedded in a dense network of positive fibres and puncta. No staining was observed at the level of the eminentia mediana.

Both types of positive neurons were observed in the dorsal thalamic region. A large cluster of type III small neurons was scattered along the nucleus dorsomedialis anterior and dorsolateralis anterior thalami (Fig. 7A). Type II and III neurons were also observed periventricularly and dorsally to the so-called nucleus paraventricularis magnocellularis. More caudally, two large groups of type II small to medium-sized neurons were observed at the level of the

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**Fig. 4.** NADPH-diaphorase system in the paleostriatal complex. A: Low-power photomicrograph (level of Fig. 2D–E) shows the differential distribution of the histochemical reaction within the paleostriatal complex of quail. B: Higher power photomicrograph of the PA shows type I multipolar neurons and the very fine network of positive processes responsible of the high intensity of staining of this region. C: Type I neurons of the PP in a region bordering the PA. The lower intensity of the staining within this region when compared with the PA is due to a lesser number of stained processes within the PP rather than to fewer stained cells. Asterisk, Lateral ventricle; PA, paleostriatum augmentatum; PP, paleostriatum primitivum. Bars = 500 μm in A, 100 μm in B and C.
nucleus dorsomedialis posterior (DMP) and nucleus ovoidalis (OV; Fig. 7B). Within the DMP the neurons were larger, multipolar, and loosely arranged, whereas, within the OV, the positive neurons were more numerous, smaller, generally triangular shaped, and embedded in a dense network of varicose fibres and puncta. In both nuclei, many scattered, small, type III neurons were also present. In the lateral thalamus, a cluster of heavily stained type II neurons was observed within the nucleus pretectalis medialis (PTM). Scattered, type II or III neurons were located around the ventral aspect of the ROT. A high density of positive fibres was observed within the anterior dorsolateral and lateral thalamic regions, corresponding to the so-called lateral geniculate complex of the pigeon (Güntürkün and Karten, 1991). A few positive cells were scattered within this wide region.

The habenular complex showed an extremely high level of innervation, mainly at the level of the HL. ND reactive fibres were so abundant as to entirely mask the structure in which neuronal cell bodies appeared almost totally negative (Fig. 7C). A lower level of density is present within the nucleus habenularis medialis, where it was also possible to observe a few neurons of the type II or III.

Optic lobes. The optic lobes of the quail contained a large number of positive neurons (Figs. 2G–H, 3A,B). Scattered, small, type III neurons were visible within the nucleus intercollicularis (ICo), which also showed a medium density positive neuropile. On the contrary, positive neurons and fibres were absent in the MLd, which is surrounded by the ICo (Fig. 8A).

Within the optic tecta, the stained neurons and fibres formed a complex pattern (Fig. 8B). The strongest reaction and the largest number of positive neurons were observed in the stratum griseum periventricularis (SGP), where several neurons of type I or II were located. Some of the marginal neurons lying just beneath the ependymal layer showed a horizontal shape, whereas the others had processes running perpendicularly towards the outer layers of the tectum.

Scattered neurons of the type II were observed at the level of layers 4 and 5 of the stratum griseum and fibrosum superficialis (SGFS; see Hunt and Brecha, 1984, for topographical description of the avian tectum). These neurons were medium-sized, fusiform or triangular, and provided with long processes vertically oriented towards the external surface of the tectum. A thin layer of positive, thin, punctate fibres covered the layers where these cells were located (Fig. 8D).

Neurons of type III were scattered in deeper layers of the SGFS (10–12). These elements were small and bipolar, with long, thin, unbranched processes (Fig. 8C). No positive fibres were observed within the more external layers (stratum opticum).

Mesencephalon, pons, and medulla. A large number of ND-positive neurons were observed within the quail brainstem (Fig. 3A–C), where they were arranged in well defined structures (Figs. 9A–F). At the mesencephalic level, positive neurons of type I were mainly located within the TP and the AVT. They were large, multipolar neurons, bearing relatively short and thick dendrites (Figs. 9A–C). Some fusiform type III neurons were also intermingled with the population of larger elements. Positive type I and II neurons were also scattered at more dorsal levels in the substantia grisea centralis and the formatio reticularis medialis mesencephali. The nucleus opticus basalis (or nucleus ectomammilaris), was, on the contrary, totally devoid of positive cell bodies. The nucleus interpunctularis (IP; Fig. 9C–D) contained many type III small elements. They were bipolar and provided with short, thin, unbranched processes. The neuropile contained a moderately dense fibre plexus. The dorsal aspect of the IP contained a denser fibre network of thinner fibres embedding negative cell bodies and a few positive neurons (Fig. 9D). The raphe region contained, down to the medulla, a moderately positive neuropile, in which there were also frequent, small, type III and scattered larger type II neurons.

Small type III and scattered, larger type II neurons, were present within the nucleus of mesencephalic root of the fifth cranial nerve (Fig. 8A). Very weakly stained type III elements were observed intermingled with negative neurons within the nucleus isthmo-opticus.

A wide population of large type I neurons was localized at the level of the LoC (mainly covering its medial aspect), whereas scattered neurons were observed within the two subcoeruleus nuclei (nucleus subcoeruleus dorsalis and ventralis; for the nomenclature in galliforms, see Guglielmone and Panzica, 1982, 1984; Fig. 9E). The neurons of these nuclei are large and multipolar, with thick, short, and branched dendrites (Fig. 9F). They were also embedded in a high density neuropile formed by thin varicose fibres and puncta.

Other prominent cell groups of the pons (Fig. 9C–E) were the nucleus pontis medialis, endowed with a great number of small type III elements (Fig. 10A), and the nucleus pontis lateralis, in which large type I and II multipolar neurons, provided with long processes were observed. Small type II and III cells were also present within this nucleus (Fig. 10B, C).

Dorsally, a heterogeneous population of ND-positive neurons was observed at the level of the vestibular nuclei (Fig. 10C). In particular, medium-sized type II neurons were located within the nucleus vestibularis dorsalis, whereas small type III and type II cells were observed around and within the nucleus vestibularis medialis. The neuropile along the median line and the ventral aspect of the pons showed a moderate to high density of innervation (Fig. 10A). Within the vestibular nuclei, only few, sparse, varicose fibres were observed. A peculiar arrangement of fibre endings was observed at the level of the nucleus laminaris, whose negative neurons were embedded on both sides by positive puncta.

The number of positive structures within the pontine region decreased moving caudally. There was a marked increase when they reached the medulla (Fig. 3E–H). Main
Fig. 6. NADPH-diaphorase positive elements in the quail ventral telencephalon (A) and diencephalon (B–F). A,B: Low-power (B) and high-power enlargement of the boundary region between the dorsal hypothalamus and the more caudal part of the PVT. A cluster of type I neurons is present in this region (level of Fig. 2E). In B, the region of the POM, as well as the whole periventricular hypothalamus, has only scattered type III elements. A group of type I neurons is located laterally to the POM and is continuous with the cluster occupying the caudal end of the PVT. C: Terminal field of positive processes in the nucleus geniculatus lateralis, pars ventralis (level of Fig. 2F) and in the decussatio supraoptica. D: Positive processes and small type III neurons in the nucleus of the decussatio supraoptica dorsalis (lateral, or visual, suprachiasmatic nucleus; see Keunzel and Masson, 1988). E,F: Low-power (E) and higher power (F) photomicrographs of the tuberal region (level between Fig. 2F and 2G). Two clusters of type III neurons (with a few element of type II or I) occupying, laterally, the LHy and medially the VMN. Neurons in the LHy (F) are less clustered than in VMN, and many of them are elongated and bipolar. CA, anterior commissure; LHy, regio lateralis hypothalami; OT, optic tract; POM, nucleus preopticus medialis; PVT, paleostriatum ventrale; VMN, nucleus ventromedialis hypothalami. Bars = 100 μm in A and C–F, 500 μm in B.
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Fig. 7. NADPH-diaphorase positive structures in the dorsal diencephalon. A: Type III neurons scattered in the nucleus dorsolateralis anterior thalami (level of Fig. 2F). B: Two of the most prominent cell groups of the region (level of Fig. 2G): the DMP, close to the ventricle, and the OV, more intensely stained. C: High intensity of staining in the habenular complex, a few type II elements are sparse within the HM. Asterisk, third ventricle; DMP, nucleus dorsomedialis posterior thalami; HL, nucleus habenularis lateralis; HM, nucleus habenularis medialis; OV, nucleus ovoidalis. Bars = 100 μm.

groups of type I and II neurons were located ventrally within the nucleus paragigantocellularis lateralis (Fig. 10E) and dorsally along the lateral aspects of the fasciculus longitudinalis medialis and within the S (Fig. 11A). Scattered type I, II, and III neurons were observed throughout the reticular formation. The type III cells were clustered around, or inside the VeM and the nuclei of ninth and tenth nerves (n IX-X). The neuropile was extremely dense within the S (Fig. 11A) and showed high density along the ventral aspect of the medulla.

The encephalic motor nuclei of the brainstem were generally unstained. Larger neurons, probably corresponding to the motor neurons, were always not stained, whereas a few, small, type III neurons were observed within the n IX-X and the motor nucleus of the 5th encephalic nerve (Fig. 11A,B). The neuropile of this last nucleus was moderately dense, showing a number of puncta surrounding large unstained cells.

The cerebellar cortex showed a weak reaction. The Purkinje cells were unstained, whereas the granule cells showed a weak reaction (type III neurons; Figs. 8A, 11C). The molecular layer was homogeneously weakly stained. Sparse networks of varicose fibres were present in the cerebellar nuclei, whose cells were generally unstained.

DISCUSSION

The distribution of ND-positive elements described in the present study appears to be unique and distinct from that of other neurochemically defined cell populations of the avian brain (for a recent review on avian peptidergic systems, see Viglietti-Panzica and Panzica, 1991; for neurotransmitters, see Dietl et al., 1988a,b; Dietl and Palacios, 1988; Ball, 1990).

Considering the recent finding reported in the Introduction that neuronal ND is an NOS (Hope et al., 1991), the present study details, for the first time in quail, the extent and topographical location of elements generating the putative messenger molecule NO. The mapping of this neural system representing one of the most prominent populations of the avian brain as well as the comparison of NO distribution in different species may provide a better opportunity to clarify its functional role in various regions of the brain.

ND-positive neurons have different morphology within the quail brain. These differences reflect the existence of various types of heavily stained neurons (long or short, branched or unbranched dendrites, multipolar or fusiform cell bodies, smooth or varicose axons) that we have classified as type I and II. Positive neurons can have a very different degree of staining. It varies, in fact, from a strong precipitate (types I and II) to a weak blue color (type III neurons). This difference was also observed in the topographical studies that were published on mammals (Mizukawa et al., 1989; Vincent and Kimura, 1992), but until now no explanation has been produced for this fact. It might be considered to be dependent on the content and/or functional activity of the enzyme in different cell groups. A few studies reported, in fact, variations occurring in this system according to different experimental situations (Sagar and Ferriero, 1987; Pow, 1992) or during regeneration (Gonzalez et al., 1987).

Olfactory pathway

The absence of a significant innervation of quail olfactory bulbs is confirmatory of important interclasses and interspecies variability at this level. In previous comparative studies on ND activity in vertebrate olfactory bulbs (Porteros, 1992), numerous ND-positive neurons were found in the olfactory bulb of Barbus meridionalis, Tinca tinca, Rana perezi, and Macaca mulatta. By a similar protocol, however, these structures were devoid of enzymatic activity in the pigeon and chicken as well as in some teleostean or reptilian
Fig. 8. Pattern of NADPH-diaphorase structures in the quail optic lobe (level of Fig. 3A). A: Low-power enlargement showing the distribution of cells and processes within the optic lobe. The MLd appears empty of both terminals and cell bodies, whereas the surrounding ICo exhibits a moderate staining of neuropile and scattered type III neurons. The arrow points to the mesencephalic radix of the fifth nerve, where positive type II and III cell bodies are localized. A large number of intensely positive neurons is distributed within the periventricular gray of the optic tectum. B: Medium-power enlargement of the TeO showing the pattern of distribution of NADPH-diaphorase from the SGP to the SO. The SO is devoid of reaction products. C: External layers of the SGFS. Scattered positive elements are distributed within these layers. A plexus of positive fibers surrounds negative elements of the layers. D: Deeper layers of the SGFS showing numerous bipolar type III elements. Ch, cerebellum; ICo, nucleus intercollicularis; IO, nucleus isthmo-opticus; Ipc, nucleus isthmi, pars parvocellularis; MLd, nucleus mesencephalicus lateralis, pars dorsalis; SAC, stratum album centrale; SGC, stratum griseum centrale; SGP, stratum griseum periventriculare; SGFS, stratum griseum et fibrosum superficialis; SO, stratum opticum; TeO, tectum opticum. Bars = 500 μm in A, 100 μm in B and D, and 50 μm in C.
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NADPH-diaphorase in the bulb (Vincent and Kimura, 1992), a structure that is largely reduced or absent in birds. In birds (Reiner and Karten, 1985) the olfactory pathway is composed of efferents terminating within the cortex pyriformis, whereas a second projection was found to terminate within the tuberculum olfactorium, and a third one was shown to enter the nucleus taeniae. This last is a component of the avian archistriatum, which is thought to be homologous to the mammalian amygdala (Zeiher and Karten, 1971). Another component of the olfactory system includes the stria medullaris and the habenular region (Reiner and Karten, 1985). With the exception of the olfactory bulb, the whole olfactory pathway thus seems to be deeply influenced by NO in quail. All the previously mentioned components, in fact, show a medium to high density of ND-positive processes and neurons.

Avian striatal complex

The telencephalic paleostriatal and paralactory lobe complex (which is, according to Kitt and Braith (1981), the avian homologue of the mammalian striatal complex) is the region that shows the highest number of intensely stained ND-positive neurons, embedded in a dense positive neuropile (extremely dense in the PA). In mammals, ND is considered a selective marker for the striatal neurons containing both NPY and somatostatin (Vincent et al., 1983a). This possibility is questionable for the quail. In fact, recent immunohistochemical studies (Aste et al., 1991) demonstrated only few NPY-positive cell bodies dispersed in a dense network of positive fibres within the paleostriatal-paralactory lobe complex of quail. On the contrary, the large majority of telencephalic ND-positive neurons was localized in the hyperstriatum as well as in the Hp. On the basis of cellular morphology, some of NADPH-positive neurons located near the lamina frontalis superior and the lamina hyperstriatrica might correspond to the NPY-positive elements detected in this region. The distribution and the morphology of cells within the neostriatum can also be compared to that of NPY-positive elements (see Fig. 2a in Aste et al., 1991). Conversely, the Hp, which is totally devoid of ND-positive cells in quail, showed a large population of NPY elements in the same species and contained ND, NPY, and somatostatin in mammals (Vincent, 1986). It is important to note that, after intraventricular colchicine injection, a wide population of NPY-positive cell bodies as well as of somatostatinergic neurons (partly colocalized) was observed in the pigeon basal ganglia (Anderson and Reiner, 1990). Further studies, under different experimental conditions, and colocalization of ND activity and NPY or somatostatin are required to confirm if in birds, as in mammals, the production of NO is a distinctive characteristic of all or part of the NPY-somatostatin-positive striatal neurons.

Septohypothalamic regions

Within the septopreoptic region of the quail the ND neurons have a peculiar distribution which is not superimposable to any of peptidergic systems studied so far (see Panzica et al., 1992). The distribution of ND neurons in the septopreoptic region of quail differs from that reported for rat (Vincent and Kimura, 1992) or cat (Mizukawa et al., 1989). In mammals, in fact, positive neurons were observed throughout the septal area, scattered both laterally as well as medially, and particularly concentrated within the nucleus of the diagonal band. In quail ND-positive neurons were highly concentrated within two specific nuclei the SM and the nCPa [which is the probable homologue of the mammalian nucleus preopticus medius, heavily labelled in the rat (Arévalo et al., 1992)]. Only scattered neurons were found within the nucleus preopticus dorsalis (and anterior), which is considered the homologue of the mammalian nucleus of the diagonal band. A few fibres and scattered, small, and weakly stained (type III) neurons were observed in the other regions.

Few neurons are stained for ND in quail hypothalamus compared with the case of mammals. In particular no staining was observed within the periventricular and lateral groups containing VT-positive cells in birds (Viglietti-Panzica, 1986) and corresponding to different subdivisions of the mammalian paraventricular and supraoptic nuclei (Sánchez et al., 1991). In mammals, a dense cluster of ND-stained neurons was detected not only in the supraoptic and paraventricular nuclei (both in magni- and in parvicellular nuclei; Vincent, 1986; Sagar and Ferriero, 1987; Arévalo et al., 1992; Pow, 1992) but also in some neurosecretory accessory nuclei such as nucleus circularis and fornical nuclei (anterior and posterior; Arévalo et al., 1992). Other accessory nuclei, such as the medial forebrain bundle nucleus, however, did not display this activity. Additionally it is currently known that, in the magnocellular neurosecretory nuclei of the rat hypothalamus, the neurons expressing ND partially coexpress calbindin (Alonso et al., 1992a), somatostatin (Alonso et al., 1992b), and vasopressin and oxytocin (Sánchez et al., 1994). This indicates that in mammals the NADPH-positive neurons are probably involved in different specific hypothalamic functions. In that there is a lack of staining of the magnocellular VT-ergic neurons in quail, the eminentia mediana did not show any ND activity, whereas it was labelled in rat (Vincent and Kimura, 1992). This lack of ND activity within the VT system of quail could underline important functional differences between quail and rat. In the latter species, in fact, recent studies (Pow, 1992) suggested functional involvement of this enzymatic system in the osmoregulation and water intake. It is of importance to note that in birds the subfornical organ and the surrounding areas are believed to be the most important for the regulation of thirst (Gerstberger et al., 1987). This region is likely to correspond to the extension of the nCPa, which was heavily stained in our preparation up to the level of the subfornical organ.

The distribution of positive neurons in rat and quail is more similar in the posterior hypothalamus. In both species two large groups of ND-positive neurons were located in the ventromedial nucleus and in the lateral hypothalamus (Vincent and Kimura, 1992). These clusters were larger in quail than in mammals and showed not only weakly stained neurons but also type II cells. This distribution partially overlaps that of aromatase-containing or estradiol-receptor bearing neurons (Balthazart et al., 1980, 1982) suggesting a probable involvement of NO in the regulation of this neuronal system devoted to the control of some reproductive activities.

Diencephalic auditory pathway

The thalamus dorsomedialis and dorsolateralis contains several groups of type II and type III positive elements scattered within the major nuclei of the region, including the PTM and the OV. Among these nuclei, the OV is particularly filled with ND positive elements. In birds this
Figure 9
Fig. 10. NADPH-diaphorase positive groups of the pons (level of Fig. 3E). A: Type III neurons of the PM. Scattered type I and II neurons are lying along the raphe region. B: Positive multipolar neurons of the PL. C: Enlargement shows the presence of both large type I and small type II neurons within the PL. D: NADPH-diaphorase reactivity within the vestibular nuclei. Several positive neurons are localized within the VeD and close to the FLM. Only scattered neurons are present within the VeM. Stars, commissura cochleaiaris dorsalis. E: Large multipolar type I neurons of the Rpgl. Type I1 and I11 neurons are scattered between the larger positive elements. FLM, fasciculus longitudinalis medialis; PL, nucleus pontis lateralis; PM, nucleus pontis medialis; R, nucleus raphe; Rpgl, nucleus reticularis paragigantocellularis lateralis; VeD, nucleus vestibularis dorsalis; VeM, nucleus vestibularis medialis. Bars = 100 μm in A, B, D, and E, 50 μm in C.

nucleus is considered part of the auditory thalamus (Karten, 1967, 1968; Durand et al., 1992) and receives a major projection from the MLd, which is analogous to the mammalian inferior colliculus (Karten, 1967; Durand et al., 1992). The MLd is, on the contrary, almost empty of positive neurons and processes. It is interesting to note that several areas listed as terminal fields of projection from the OV in many avian species (for references see Durand et al., 1992) show, in quail, a rich supply of positive processes. In particular this is characteristic of the PVT and of the neostriatum caudale [even if the field L, considered the avian homologue of the mammalian auditory cortex (Karten, 1967, 1968) does not show any peculiar accumulation of positive processes]. It thus seems that the avian auditory pathway is, at least partly, regulated by NO.

Optic system

The optic lobes contain a complex pattern of ND neurons, both in the periventricular layers (ICo and SGP) and in the...
more superficial layers of the tectum. Within the tectum the cells are scattered in many layers of the SGFS, whereas only a few cells are located in the stratum griseum centralis. In the chicken, those latter neurons were described as solitary magnocellular elements corresponding to part of the stellate ganglion cells (Martínez-de-la-Torre et al., 1987). The wide innervation of the optic tecta suggests a deep involvement of NO in their regulation. A large number of positive neurons was described in the rat superior colliculus, the mammalian homologue of the avian optic tectum. These neurons formed a complex laminar pattern within the colliculus (Vincent and Kimura, 1992; González-Hernández et al., 1992). In mammals ND neurons are present at all levels of the visual system, suggesting that NO plays an important role in regulating visual functions (González-Hernández et al., 1992; Mitrofanis, 1992). The situation seems in some sense comparable to that in birds. The ND technique has revealed particular populations of retinal interneurons (amacrine cells) in pigeon (Sato, 1990), and of scattered magnocellular neurons in the chicken optic tecta (Martínez-de-la-Torre et al., 1987). In the present study, we detailed the complex pattern of distribution of ND neurons within different layers of the quail optic tecta and SCN1. The whole lateral geniculate complex (Gün-türkün and Karten, 1991), considered at present as the most important retinorecipient area, has medium to high levels of innervation and is provided by scattered positive neurons. The optic tecta (Hunt and Brecha, 1984) and SCN1 are part of the tectofugal optic pathway in birds. These nuclei project to some subecentral nuclei, including the ROT (Karten and Hodos, 1970; Hodos et al., 1982), which in turn project to the E (considered the avian homologue of the mammalian visual cortex, Hodos et al., 1982; Karten, 1991). These last two nuclei contain no ND-positive fibres and cells. The lateral geniculate complex is part of the thalamofugal visual pathway, and sends efferents to the wulst [which includes several nuclei of the hyperstriatum (Karten et al., 1973)]. In the latter region, ND-positive fibres and neurons were observed. From the wulst, efferents project to the ectostriatal region. These findings suggest that NO plays an important role in the regulation of avian primary visual centers.

**Mesopontine system**

A large population of ND-containing neurons has been observed in the mesopontine region of the Japanese quail. Their distribution (AVT, IP, TP, LoC, nuclei subcoerulei) overlaps that of the quail catecholaminergic system described both histochemically (FAGLU method; Panzica et al., 1991a) and immunohistochemically (detection of the enzymes tyrosine hydroxylase and dopamine β-hydroxylase; Bailhache and Balthazart, 1993). There is, however, no relevant relationship with the serotoninergic system (Cozzi et al., 1991). The cell typology also closely resembles that of avian catecholaminergic neurons (Guglielmone and Panzica, 1982, 1984; von Bartheld and Bothwell, 1992). In rat and in cat, ND-positive neurons are distinct from

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**Fig. 11.** A: Dense reaction for NADPH-diaphorase within the neuropile of the nucleus of the solitary tract (S, level of Fig. 3G). Scattered neurons are present within the nucleus of the vagus nerve (X) and in the nucleus vestibularis medialis (VeM). Large neurons are clustered laterally to the fasciculus longitudinalis medialis (FLM). B: Motor nucleus of the fifth nerve. Small type III positive neurons are scattered between the large negative motor neurons of the nucleus. C: High magnification (interferential contrast) of the cerebellar cortex. The Purkinje cells (P), as well as the white matter (W), are negative for NADPH-diaphorase reaction, whereas the granule cells (G) are moderately positive. A diffuse reaction is present in the molecular layer (M). Asterisk, ventricle; stars, commissura cochleaearis dorsalis. Bars = 100 μm.
catecholamine-synthesizing elements and are likely to correspond to the cholinergeric neurons of the pontomesencephalic tegmentum (Vincent et al., 1983b; Reiner and Vincent, 1987; Mizukawa et al., 1989). In most mammalian species, pontomesencephalic noradrenergic and cholinergeric neurons are localized in distinct nuclei, with noradrenergic neurons concentrated within the LoC and cholinergeric neurons within the laterodorsal tegmental and pedunculopontine nuclei (Mesulam et al., 1983). In the cat, noradrenergic and cholinergeric (also ND positive) neurons are, on the contrary, extensively intermingled in the brainstem tegmentum (Reiner and Vincent, 1987). Thus, in mammals, ND is considered a specific marker for the mesopontine cholinergeric system (Vincent et al., 1983b).

A detailed description of avian mesencephalic cholinergeric system was provided by the study of Sorenson et al. (1989) in the chicken. In that study, a large supply of ChAT-immunopositive fibres was observed in the IP as well as in several mesencephalic location. Small ChAT-positive cells were observed in the optic tecta, whereas large cells were found in various areas of the tegmentum, in particular in the TP. Even if not described in the text, cells were present in the region of the LoC (see Figs. 22–24 of Sorenson et al., 1989). More recently, von Bartheld and Bothwell (1992) demonstrated in a developmental study that noradrenergic and cholinergeric neurons are intermingled in the chicken LoC as well as in the subcoerulean nuclei. The present data in quail do not clarify whether ND is contained in both cell types or is confined in the cholinergic population as appears in mammals. Double-staining investigations will clarify whether or not, as in mammals, these two systems are largely coexistent. Preliminary investigations performed in our laboratories with NH histochimistry and tyrosine hydroxylase immunocytochemistry (employing a commercial antibody from ETI, Eugene, OR) demonstrated that a small fraction of tyrosine hydroxylase-immunoreactive elements shows ND activity.

In conclusion, the present histochemical observations confirm that the neuronal system positive for ND, and probably involved in the production of NO, is widely diffused not only in mammals but also in nonmammalian species. In quail, it appears that this system has a development and an importance similar to that suggested for rodents. To a large extent, the distribution of such an enzyme appears comparable in both rat and quail. However, interspecific differences have been observed in some important sites (e.g., the magnocellular hypothalamic system, the olfactory bulb), suggesting that different neuronal circuits can be involved in the control of similar functions in different phyla.

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